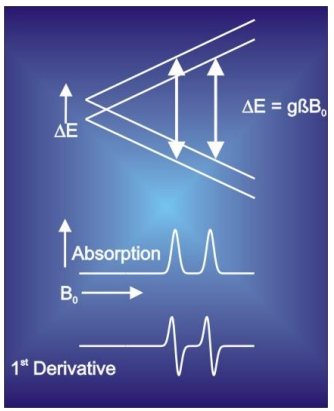


Electron Paramagnetic Resonance Theory

E.C. Duin

EPR, the Technique….

- Molecular EPR spectroscopy is a method to look at the structure and reactivity of molecules.
- EPR is ***limited to paramagnetic substances*** (unpaired electrons). When used in the study of metalloproteins not the whole molecule is observed but only that small part where the paramagnetism is located.
- This is usually the central place of action – the active site of enzyme catalysis.
- Sensitivity: 10 μM and up.
- Naming: Electron paramagnetic resonance (EPR), electron spin resonance (ESR), electron magnetic resonance (EMR)

Wilfred R. Hagen

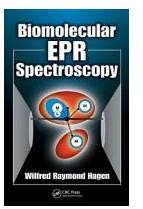
Paper:

EPR spectroscopy as a probe of metal centres in biological systems (2006) Dalton Trans. 4415-4434

Book:

Biomolecular EPR Spectroscopy (2009)

Fred Hagen completed his PhD on EPR of metalloproteins at the University of Amsterdam in 1982 with S.P.J. Albracht and E.C. Slater.



Spectral Simulations

- The book 'Biomolecular EPR spectroscopy' comes with a suite of programs for basic manipulation and analysis of EPR data which will be used in this class.
- Software: www.bt.tudelft.nl/biomolecularEPRspectroscopy

Isotropic Radicals


Simple Spectrum

Hyperfine Spectrum

EPR File Converter

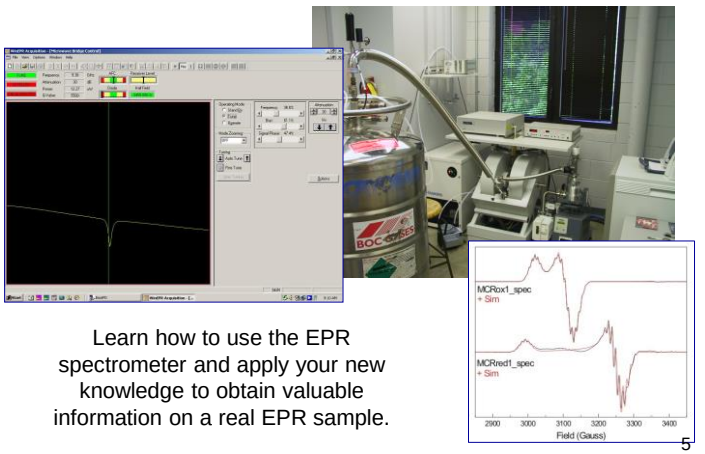

EPR Editor


Single Integer Signal

GeeStrain-5


Visual Rhombo

BYOS (Bring Your Own Sample)



Learn how to use the EPR spectrometer and apply your new knowledge to obtain valuable information on a real EPR sample.

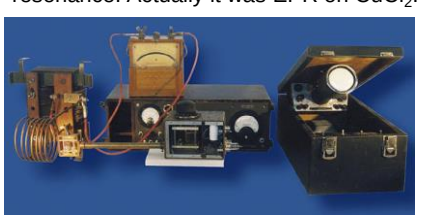
EPR Theory

A Free Electron in Vacuo

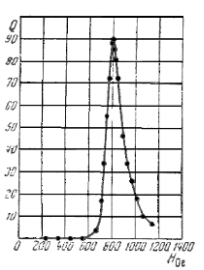
Free, unpaired electron in space:
electron spin - magnetic moment

Discovery

In 1944, E.K. Zavoisky discovered magnetic resonance. Actually it was EPR on $CuCl_2$.

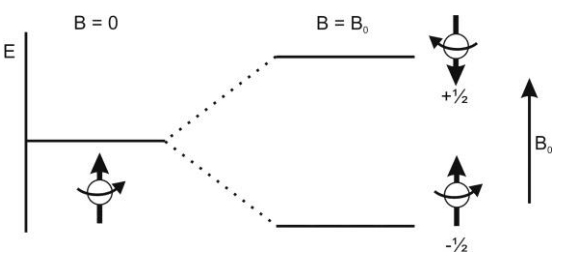


E.K. Zavoisky's first EPR system



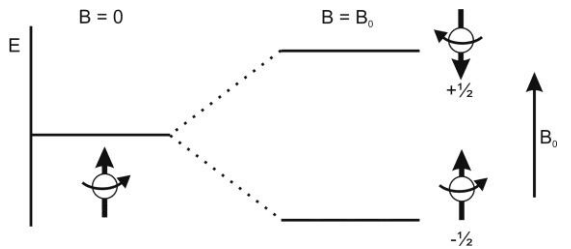
First EMR on $CuCl_2.2H_2O$
4.76mT @ 133MHz

A Free Electron in a Magnetic Field



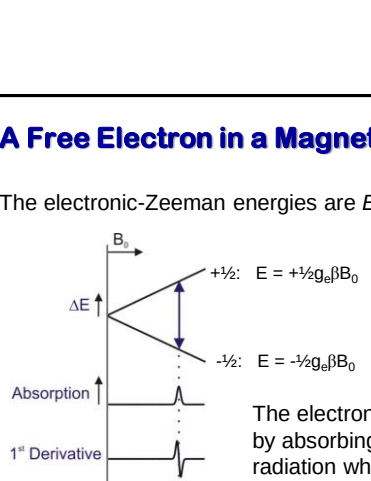
- An electron with spin $S = ½$ can have two orientations in a magnetic field B_0 labeled by $m_S = +½$ or $m_S = -½$.
- The unpaired electron will have a state of lowest energy when the moment of the electron is aligned with the magnetic field and a stage of highest energy when aligned against the magnetic field.

A Free Electron in a Magnetic Field



- The energy of each orientation is the product of μ and B_0. For an electron $\mu = m_s g_e \beta$, where β is a conversion constant called the Bohr magneton and g_e is the spectroscopic *g*-factor of the free electron and equals 2.0023192778 (≈ 2.00). Therefore, the energies for an electron with m_s = +½ and m_s = -½ are, respectively: ½ $g_e \beta B_0$ and -½ $g_e \beta B_0$

A Free Electron in a Magnetic Field



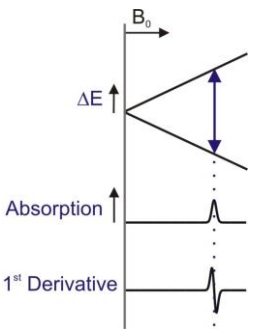
The electronic-Zeeman energies are $E = m_S g \beta B$

The electron can change orientation by absorbing electromagnetic radiation which energy should exactly equal the state energy difference ΔE, and this defines the resonance condition:

$$\Delta E = h\nu = g_e \beta B_0$$

A Free Electron in a Magnetic Field

$$h\nu = g_e \beta B_0$$



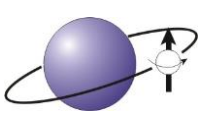
Two fundamental constants:
- Planck's constant: *h*
- Bohr magneton: β

Two experimental parameters:
- Microwave frequency: *v*
- Magnetic field: B_0

A constant of proportionality: ***g-value***

Property of matter, for the free electron: $g = g_e = 2.00232$

Spin-orbit Coupling



Resonance condition: $h\nu = g_e \beta B_0$

When the electron is bound to one, or more nuclei, then a virtual observer on the electron would experience the nucleus (nuclei) as an orbiting positive charge producing a second magnetic field, δB, at the electron.

$$h\nu = g_e \beta (B_e + \delta B)$$

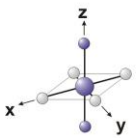
Since only the spectrometer value of *B* is known:

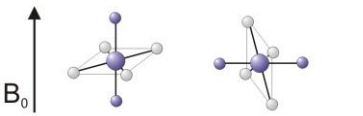
$$h\nu = (g_e + \delta g)\beta B = g \beta B$$

The quantity $g = g_e + \delta g$ contains the chemical information on the nature of the bond between the electron and the molecule, the electronic structure of the molecule.

The value of *g* can be taken as a fingerprint of the molecule.

Anisotropy





$g = g_e + \delta g_z$ $g' = g_e + \delta g_{xy}$

Anisotropy: the fact that molecular properties, such as δg are angular dependent and reflect the 3D electronic structure of the paramagnet.

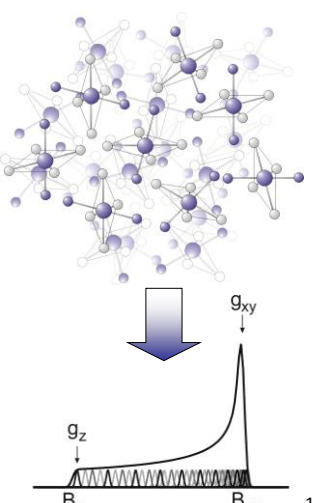
Example: compound with axial paramagnetic anisotropy. This will have a different δg-value for different orientations dependent on the alignment of B_0 along the *z*-axis or the *y*- or *x*-axes.

Powder Spectrum

A sample of realistic size consists of randomly oriented molecules, resulting in a so-called ***powder spectrum***.

In the example of the compound with axial paramagnetic anisotropy, the spectrum has axial EPR absorption.

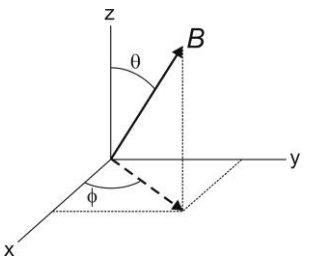
(Higher chance of having the *B*-vector anywhere in the *xy*-plane than parallel to the *z*-axis.)



Angular Dependency of *g*-Value

Defining the orientation of the magnetic field (a vector) with respect to the coordinates of the molecule (and vice versa).

Two polar angles, θ and ϕ, where θ is the angle between the vector *B* and the molecular *z*-axis, and ϕ is the angle between the projection of *B* onto the *xy*-plane and the *x*-axis.



In practice so-called direction cosines are used:

$l_x = \sin\theta \cos\phi$
$l_y = \sin\theta \sin\phi$
$l_z = \cos\theta$

Angular Dependency of *g*-Value

Resonance: $B_{res} = \dfrac{h\upsilon}{g(l_x, l_y, l_z)\beta}$

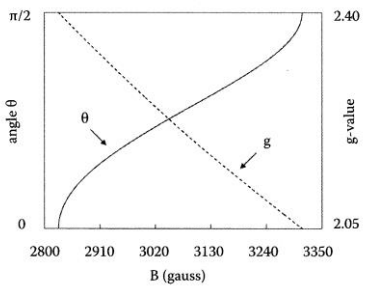
with $g(l_x, l_y, l_z) = \sqrt{l_x^2 g_x^2 + l_y^2 g_y^2 + l_z^2 g_z^2}$

In terms of the polar angles:

$$g(\theta, \phi) = \sqrt{g_x^2 sin^2\theta cos^2\phi + g_y^2 sin^2\theta sin^2\phi + g_z^2 cos^2\theta}$$

Angular Dependency of *g*-Value

For axial spectra: $g_{ax}(\theta) = \sqrt{g_{xy}^2 sin^2\theta + g_z^2 cos^2\theta}$



Plot of the angle θ and the axial *g*-value versus the resonance field (g_z = 2.40 and g_{xy} = 2.05, v = 9500 MHz)

g-Value

$$h\nu = g\beta B_0$$

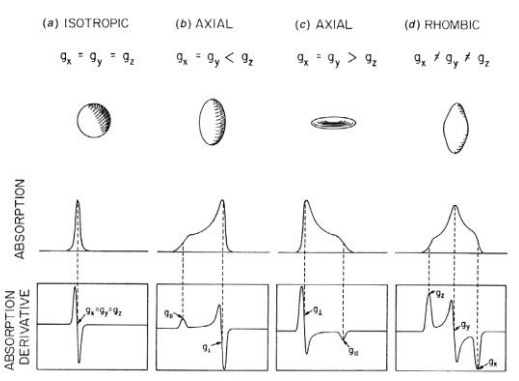
Two fundamental constants:

- Planck's constant: h
- Bohr magneton: β

For X-band:

$$g = 0.7145 \frac{\nu\ (MHz)}{B\ (Gauss)}$$

Line Shape of EPR Spectra

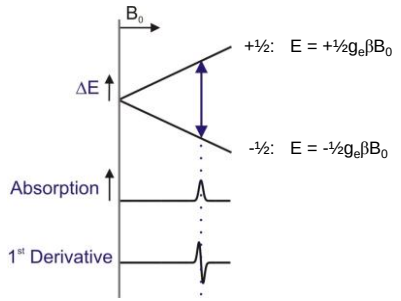


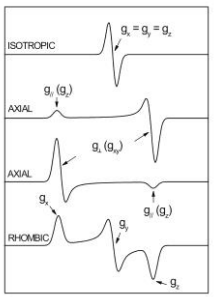
Note: $g_{//} = g_z$ and $g_\perp = g_{xy}$

Correlation Between Line Shape and Structure?

- Does the shape of the EPR signal, 'isotropic', 'axial', or 'rhombic' reflect the symmetry of a coordination site in a metalloprotein?
- Most of the time the answer is: No!
- Example: the Cu(II) spectrum of plastocyanin is virtually axial ($g_x \approx g_y$) even when recorded at higher frequency for increased *g*-value resolution.
- Crystallographic analysis, however, reveals a highly distorted tetrahedral site essentially with no symmetry at all!

S=1/2 Systems





Resonance condition:

$$\Delta E = h\nu = g_e \beta B_0$$

For X-band:

$$g = 0.7145 \frac{\nu\,(MHz)}{B\,(Gauss)}$$

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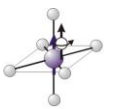
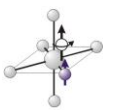
Hyperfine Interactions

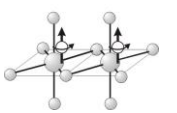
Additional splitting can be observed in EPR signals:



Hyperfine interaction
Interaction of the electron spin with the nuclear spin of the metal ion nucleus



Super hyperfine interaction
Interaction of the electron spin with first coordinate sphere ligands nuclei



Spin-spin interaction
Interaction of the electron spin with other electron spins within 10 Å distance.

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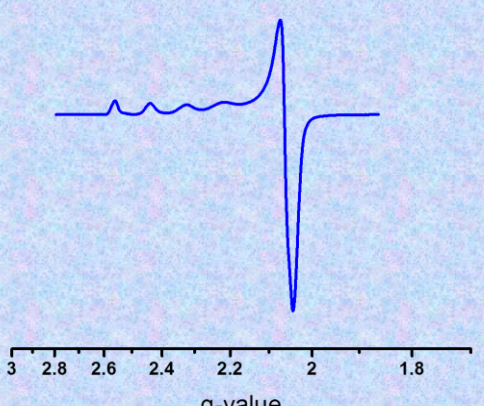
What is this !?!



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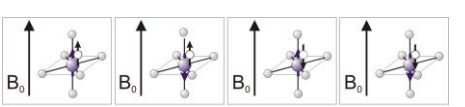
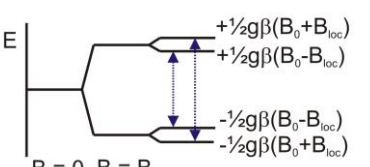
Hyperfine Interactions





- Interaction of the electron spin (S = ½) with the nuclear spin of the metal ion nucleus (I = ½)
- Four different situations: Four new energy levels

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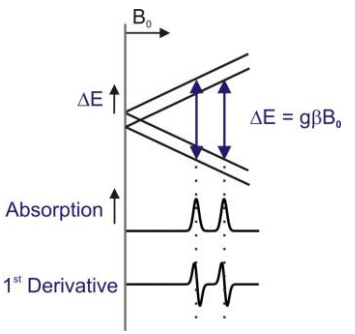
Hyperfine Interactions

- With the four new energy levels there are two field positions where the resonance conditions are met.
- The signal is in principle split in two.
- The original, unsplit, peak would have been exactly in the middle of the two hyperfine lines.



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Quantum Mechanical Description

- A full quantum mechanical description of the spectroscopic EPR event is not possible due to the complexity of the systems under study.
- In EPR we use the concept of the ***spin Hamiltonian***. This describes a system with an extremely simplified from of the Schrödinger wave equation that is a valid description only of the lowest electronic state of the molecule plus magnetic interactions.

$$H_s\psi_s = E\psi_s$$

With: H_s, spin Hamiltonian; ψ_s, the spin functions; E, energy values of the ground state spin manifold.

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Quantum Mechanical Description

- For an isolated system with a single unpaired electron and no hyperfine interaction the only relevant interaction is the electronic Zeeman term, so the spin Hamiltonian is

$$H_s = \beta B(g_x l_x S_x + g_y l_y S_y + g_z l_z S_z)$$

A shorter way of writing this is

$$H_s = \beta B \bullet g \bullet S$$

Solving this we get the equation we saw earlier for the angular dependency of the *g*-value

$$h\upsilon = \sqrt{g_x^2 l_x^2 + g_y^2 l_y^2 + g_z^2 l_z^2}\ \beta B$$

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Quantum Mechanical Description

- More terms can be added to the Hamiltonian when needed.
- For hyperfine interactions H_s becomes

$$H_s = \beta B \bullet g \bullet S + S \bullet A \bullet I$$

where *A* is the anisotropic hyperfine tensor.

- For multi-electron (high-spin) systems H_s becomes

$$H_s = \beta B \bullet g \bullet S + S \bullet D \bullet S$$

where *D* is the zero-field interaction.

- When both are present, both terms will have to be added!

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Quantum Mechanical Description

- These simplified wave equations will sometimes, under strict conditions, give analytical solutions.
- *It is important to realize that a lot of the tools and simulations software used in biomolecular EPR spectroscopy can only be used when certain conditions are met.*
- *In most systems we will encounter, we can use these tools without any problem. There are specific cases, however, where we cannot use these tools.*

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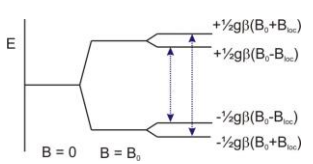
Quantum Mechanical Description

- Hyperfine interaction: $H_s = \beta B \bullet g \bullet S + S \bullet A \bullet I$



- ***Assumption 1***: the Zeeman interaction is much larger (two orders of magnitude or more) than the hyperfine interaction which can therefore be treated as a perturbation of the larger Zeeman interaction.
- ***Assumption 2***: Just like the Zeeman interaction, the hyperfine interaction will be anisotropic. It is assumed that *g* and *A* are collinear.

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Quantum Mechanical Description

- Using these assumptions the resonance condition becomes

$$h\upsilon = g\beta B_0 + hAm_I$$

where ***A*** is called the ***Hyperfine Coupling Constant*** and m_I is the magnetic quantum number for the nucleus.

- This describes most hyperfine patterns we will encounter.
- Exceptions can be found for example for Cu-ion spectra (*A*-values of 30-200 Gauss) measured at lower frequencies (L-band). In some Cu spectra the *g* and *A* tensors are not linear.
- Other examples are Mn^{2+} spectra where *D* is small.

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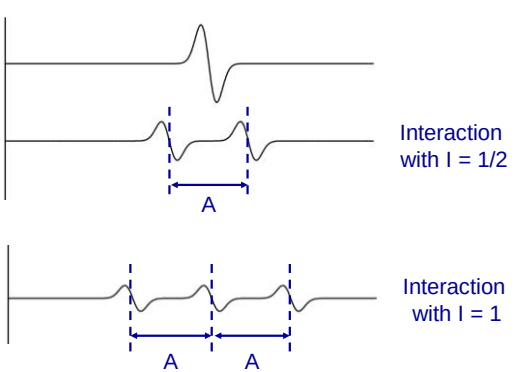
Hyperfine Interactions

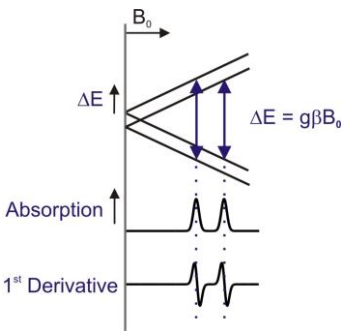
- Hyperfine coupling constant A



32

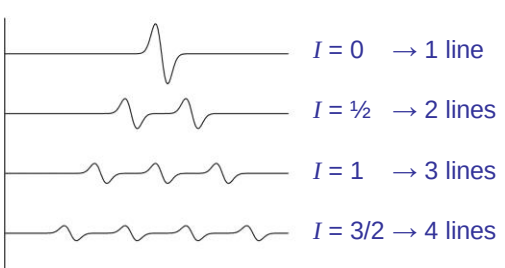
Hyperfine Interactions

- With the four new energy levels there are two field positions where the resonance conditions are met.
- The signal is in principle split in two.
- The original, unsplit, peak would have been exactly in the middle of the two hyperfine lines.

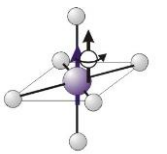


Hyperfine Interactions

- Since there are $2I + 1$ possible values of m_I ($m_I = I, I\text{-}1, \ldots, 0, \ldots, -I\text{+}1, -I$), the hyperfine interaction terms splits the Zeeman transition into $2I + 1$ lines of equal intensity.



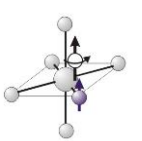
Hyperfine Interactions



- Bio transition metal nuclear spin and their *hyperfine* structure

Metal	Valency	Isotope	Spin (abundance)	EPR lines
V	IV	51	7/2	8
Mn	II	55	5/2	6
Fe	III	54, 56, *57*, 58	0 + *1/2* (2%)	1 + 2 (1%)
Co	II	59	7/2	8
Ni	III,I	58, 60, *61*, 62, 64	0 + *3/2* (1%)	1 + 4 (0.25%)
Cu	II	63, 65	3/2	4
Mo	V	92, 94, *95*, 96, *97*, 98, 100	0 + *5/2* (25%)	1 + 6 (4%)
W	V	180, 182, *183*, 184, 186	0 + *1/2* (14%)	1 + 2 (7%)

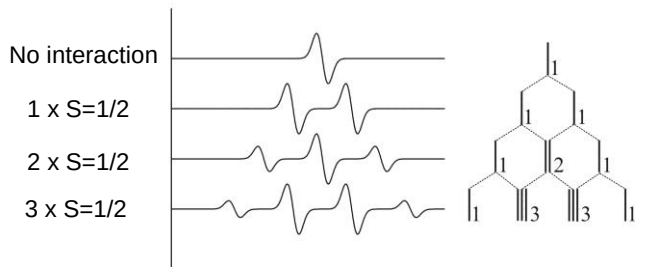
Hyperfine Interactions



Bio ligand atom nuclear spins and their EPR *superhyperfine* patterns

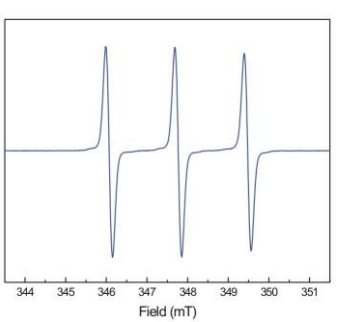
Ligand	Isotope	Spin (abundance)	EPR lines
H	1, 2	1/2 + 1 (0.015%)	2 + 3
C	12, *13*	0 + *1/2* (1.1%)	1 + 2
N	14, *15*	1 + *1/2* (0.4%)	3 + 2
O	16, *17*, 18	0 + *5/2* (0.04%)	1 + 6
F	19	1/2	2
P	31	1/2	2
S	32, *33*, 34	0 + *3/2* (0.8%)	1 + 4
Cl	35, 37	3/2	4
As	75	3/2	4
Se	76, *77*, 78, 80, 82	0 + *1/2* (7.6%)	1 + 4
Br	79, 81	3/2	4
I	127	5/2	6

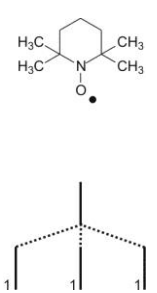
Hyperfine Interactions



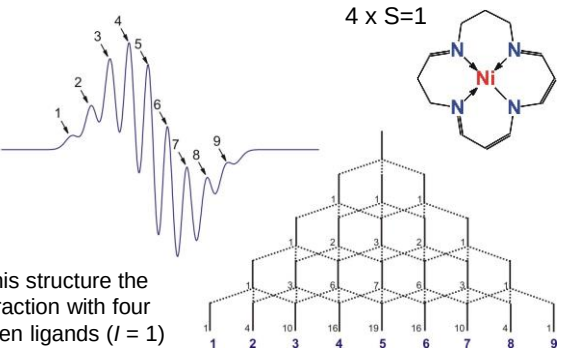
The splitting pattern (amount of peaks and relative peak intensity) can be obtained via a stick diagram or Pascal's triangle.

Hyperfine Interactions: TEMPO



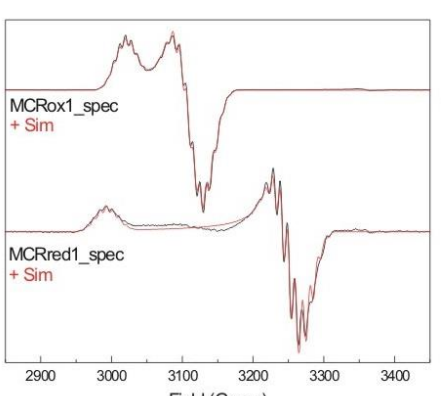


Hyperfine Interactions: MCR



In this structure the interaction with four nitrogen ligands (I = 1) would result in 9 superhyperfine lines.

Hyperfine Interactions: MCR



Type Identification - Metals

- Which redox state is EPR active?

Metal Ion	Electron Configuration	Spin State
Fe^{2+}	d^6	S = 0 (ls) or S = 2 (hs)
Fe^{3+}	d^5	S = 5/2 (hs)
Ni^{1+}	d^9	S = ½
Ni^{2+}	d^8	S = 0 or S = 1
Ni^{3+}	d^7	S = ½
Cu^{1+}	d^{10}	S = 0
Cu^{2+}	d^9	S = ½

Prepare different samples: 1) as such
2) reduced (dithionite)
3) oxidized (ferricyanide)

- How many unpaired electrons? Different spin states!

Type Identification - Metals

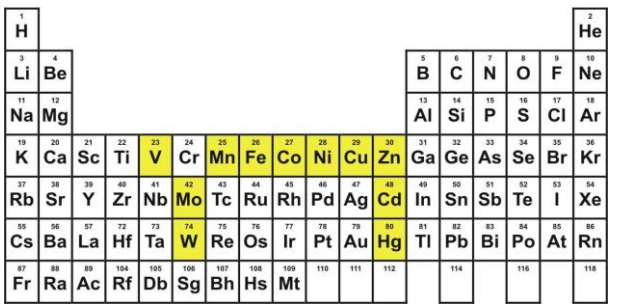
- Has the metal a nuclear spin?

Atom	Isotope	Spin (abundance)
V	50, 51	^{50}V, 6 (0.25); ^{51}V, $^7/_2$ (99.75)
Mn	55	$^5/_2$
Fe	54, 56, 57, 58	$^1/_2$ (2.119)
Co	59	$^7/_2$
Ni	58, 60, 61, 62	$^3/_2$ (1.14)
Cu	63, 65	^{63}Cu, $^3/_2$ (69.17); ^{65}Cu, $^3/_2$ (30.83)
Mo	92, 94, 95, 96, 97, 98, 100	^{95}Mo, $^5/_2$ (15.92); ^{97}Mo, $^5/_2$ (9.55)
W	180, 182, 183, 184, 186	$^1/_2$ (14.3)

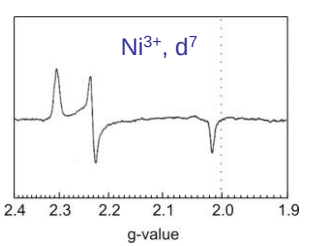
Is the signal going to be split into 2 I + 1 lines?

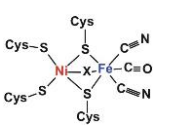
- In general: The spin–orbit coupling parameter is positive for systems with less than half filled outer shells and negative for those with more than half filled shells, which means that the former have $g<g_e$ and the latter have $g>g_e$.

Type Identification - Metals



Nickel



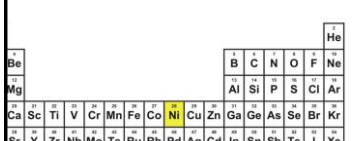


Hydrogenase.

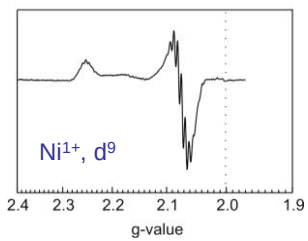
The iron is low-spin Fe^{2+}

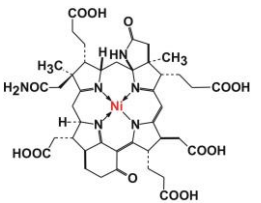
The signal is due to the nickel

$g>g_e$: g_{xyz} = 2.32, 2.24, 2.01



Nickel

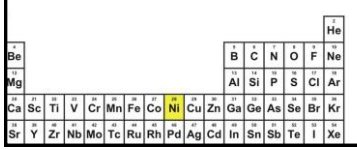




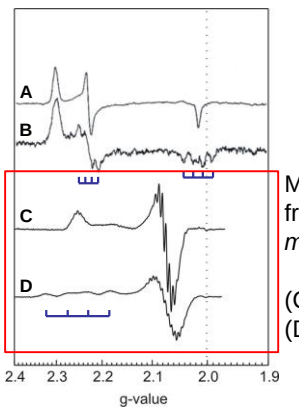
Methyl-coenzyme-M reductase

$g>g_e$: g_{xyz} = 2.252, 2.073, 2.064

Hyperfine structure due to N-ligands detectable



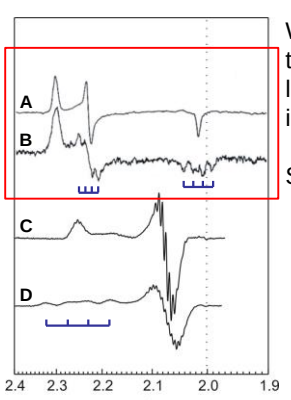
Type Identification - Nickel



Methyl-coenzyme-M reductase from *Methanothermobacter marburgensis*

(C) natural Ni
(D) growth in the presence of ^{61}Ni (86 %)

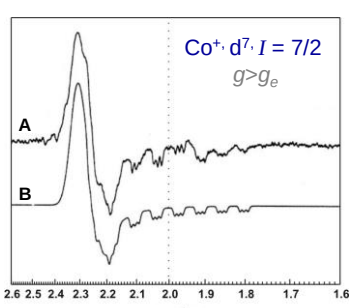
Type Identification - Nickel

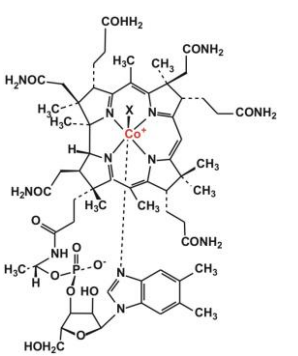


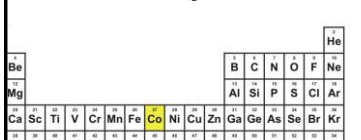
What if you are not certain about the origin of the spectrum: labeling studies with nuclear isotopes

Spectra of hydrogenase
(A) growth was performed with natural Ni (natural abundance of ^{61}Ni is 1.19%);
(B) growth in the presence of ^{61}Ni (I = 3/2)

Cobalt

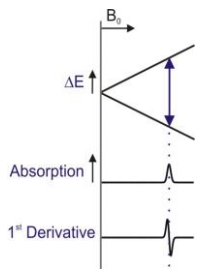






Methyltransferase from *M. marburgensis*. (**A**) Protein as isolated. (**B**) Computer simulation. g_{xyz} = 2.2591, 2.2530, 2.00659

The Microwave Frequency

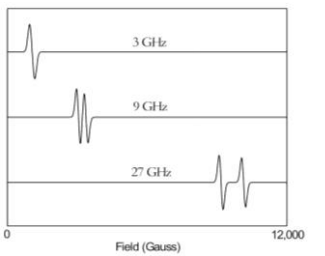


- An increase in the strength of the magnetic field B_0 will result in a larger separation of the two energy levels.
- As a result there will be an increased population difference between the ground and excited state resulting in higher signal amplitude.
- To be able to meet the resonance conditions the frequency will also have to be increased according to

$$g = 0.7145 \frac{\nu\ (MHz)}{B\ (Gauss)}$$

The Microwave Frequency

- EPR absorption lines can have a width that is independent of the used frequency and the corresponding resonance field. As a consequence, the resolution of two partially overlapping lines will increase with increasing frequency.



- *Note that there is a theoretical limit of maximal resolution enhancement by frequency increase. In practical cases the enhancement is usually less or in some cases there is no enhancement at all.*

The Microwave Frequency

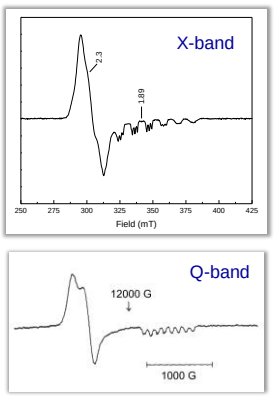
- Starting at 133 MHz just like NMR spectroscopy researchers have been pushing to get better resolution and better sensitivity.
- For both technical and fundamental reasons it turned out that the optimum sensitivity in EPR is reached in the 8-12 GHz range and X-band is right there in the middle of that range.
- There are cases, however, that the information obtained at X-band frequencies is limited and a higher frequency is needed.

The Microwave Frequency

- The Zeeman interaction is field dependent
- The linewidth is generally not field dependent (with the exception of *g*-strain).
- The (super) hyperfine interactions are also independent of the magnetic field.
- Therefore, changing the microwave frequency means changing the relative weight of the *B*-dependent and *B*-independent interactions and so the shape (and information content) of the spectrum changes with frequency.
- Note that by doing this, for example, the description of the high-spin systems is no longer valid.

The Microwave Frequency

- Comparison of a Vitamin B_{12} spectrum at X-band (9.5 GHz) and Q-band (35 GHz).
- In this example, the superhyperfine lines are still resolved at Q-band, but are not overlapping with the g_x- and g_y-peaks anymore. Now the 8 hyperfine lines can be detected without any problems



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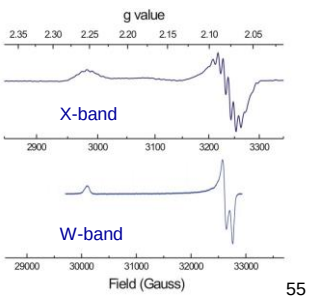
The Microwave Frequency

- When the spectra are both plotted on the same *g*-scale is seems like the linewidth is much smaller, and small differences in *g*-values can be detected.
- In this example, the superhyperfine lines are not resolved at W-band.



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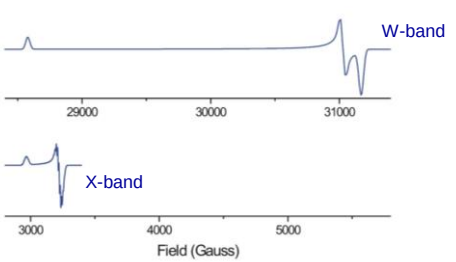
The Microwave Frequency

- Comparison of the MCRred1 spectrum at X-band (9.5 GHz) and W-band (90 GHz).
- Note that the linewidth does not change on a linear Gauss-scale (both scales cover 3000 Gauss).



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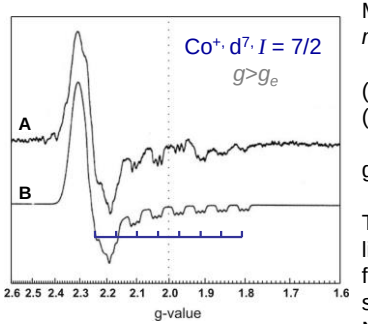
Cobalt

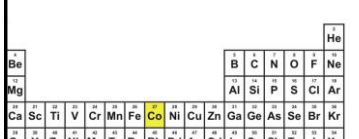


Methyltransferase from *M. marburgensis*.

(A) Protein as isolated.
(B) Computer simulation

g_{xyz} = 2.2591, 2.2530, 2.00659

The position of the 8 hyperfine lines are indicated in the figure. Each line, in turn, is split due to interaction with a N-ligand



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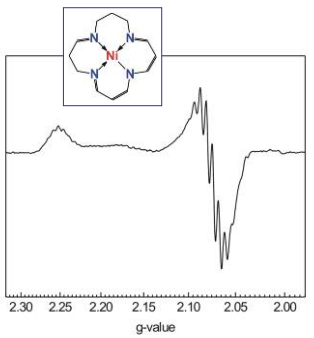
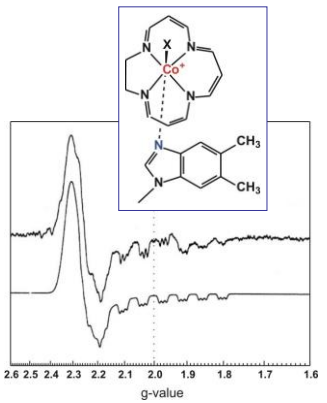
Identification of Ligands

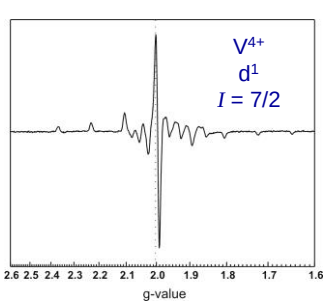


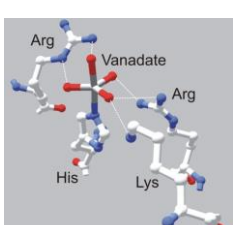
Free electron in $d_{x^2-y^2}$ orbital



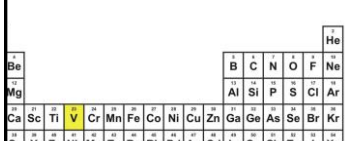
Free electron in d_{z^2} orbital

Vanadium





Vanadium-containing chloroperoxidase from the fungus *Curvularia inaqualis*

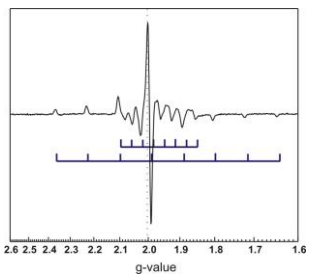


Vanadium

Spectrum is due to V^{4+}
d^1, $I = 7/2$

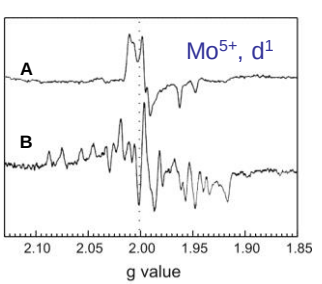
The spectrum is axial:
$g_{//} = 1.95$ and $g_{\perp} = 1.98$ ($g<g_e$)

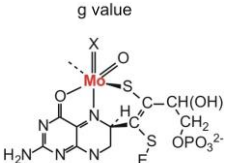
Both lines are split into 8 hyperfine lines.



The hyperfine splitting *A* is very large and the hyperfine lines of one peak will be on the other side of the other peak. This causes an effect called ***overshoot***: The orientation and shape of these lines will change.

Molybdenum



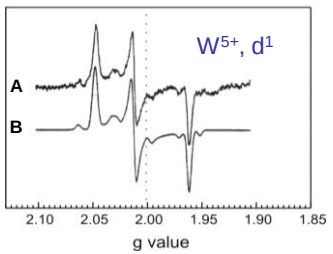


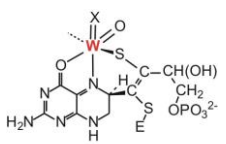
Methanobacterium wolfei formyl-methanofuran dehydrogenase (FDH I) isolated from cells grown on molybdate

(A) Two signals with g_{xyz} = 2.003, 1.989, 1.955 and g_{xyz} = 2.00, 1.984, 1.941
(B) Cells grown in the presence of ^{97}Mo-molybdate ($I = 5/2$).

Note that we have *g*-values above and below g_e

Tungsten





E = H, CH_3 or protein

Formyl-methanofuran dehydrogenase (FDH II) from cells grown on tungstate.

(A) g_{xyz} = 2.0488, 2.0122, 1.9635.
(B) Simulation of C based on the natural abundance of the tungsten isotopes: I = 0: ^{180}W, 0.14%; ^{182}W, 26.4%; ^{184}W, 28.4% and I = 1/2: ^{183}W, 14.4%.

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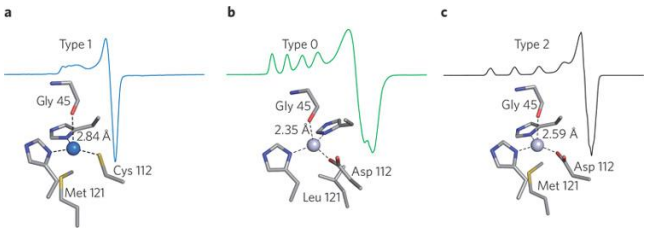
Copper



Comparison of EPR spectra and structures for copper centres in azurin. a, Type 1 (wildtype). b, Type 0 (C112D/M121L). c, Type 2 (C112D).
(Rosenzweig (2009) Nature Chemistry 1, 684 – 685)

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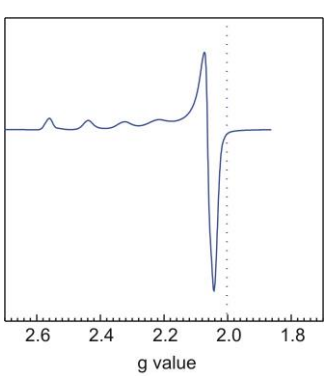
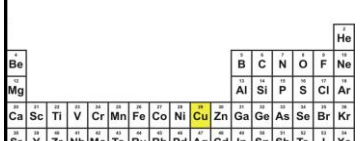
Copper

Cu^{2+} in $Cu(ClO_4)_2$
d^9, I = 3/2
Axial signal
(g>g_e)





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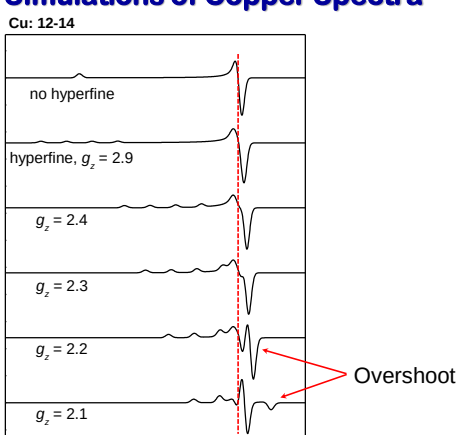
Simulations of Copper Spectra



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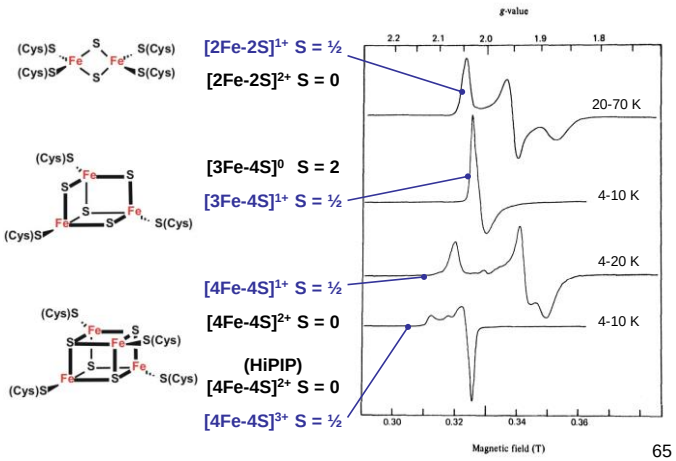
Iron-sulfur Clusters



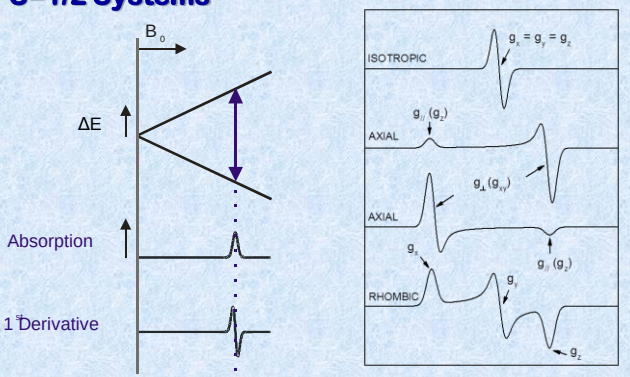
High-Spin Systems

- A system with *n* unpaired electrons has a spin equal to *S* = *n*/2. Such a system has a spin multiplicity:

$$m_s = 2S + 1$$

- This value is equal to the number of spin energy levels.
- All the spin levels together are called the *spin multiplet*.
- An essential difference between S = ½ systems and high-spin or S ≥ 1 systems is that the latter are subject to an extra magnetic interaction namely between the individual unpaired electrons.
- Unlike the electronic Zeeman interaction this interaction is always present and is independent of any external field. Another name for this interaction therefore is *zero-field interaction*.

S=1/2 Systems



Resonance condition:

$$\Delta E = h\nu = g_e \beta B_0$$

(non-)Kramers' Systems

Systems with more than one unpaired electron

Half-integer / Kramers' systems

- *S* = 3/2, 5/2, 7/2, 9/2
- All systems detectable in ***perpendicular-mode EPR***

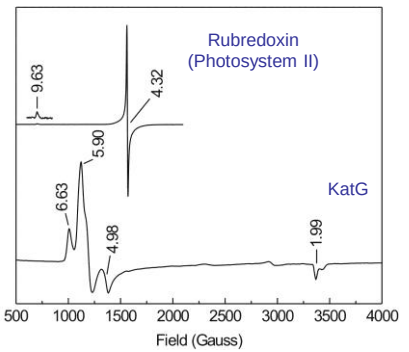
Integer / non-Kramers' systems

- *S* = 1, 2, 3, 4
- Detection in ***parallel-mode EPR***
- In biochemistry only relevant for *S* = 2 systems of $[3Fe\text{-}4S]^0$ and Heme-Fe^{2+}

Examples for Fe^{3+}/S = 5/2

Example 1: Fe^{3+} in rubredoxins. The ion is coordinated by the thiolate groups of four Cys residues.

Example 2: Fe^{3+} in the heme group of KatG. The ion is coordinated by the four nitrogen ligands from the heme ring.



Half-Integer / Kramers' systems

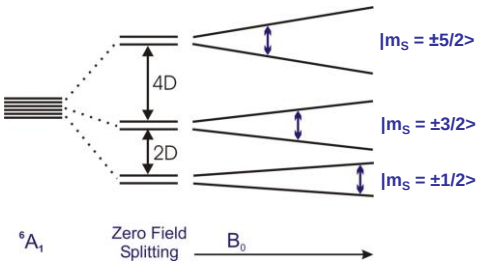
- The spin Hamiltonian becomes

$$H_s = \beta B \bullet g \bullet S + S \bullet D \bullet S$$

- Solving the wave equations it can be shown that in zero field, the sub levels of a half-integer spin multiplet group in pairs (***Kramer pairs***) and these pairs are separated by energy spacings significantly greater than the X-band microwave energy *hv*.

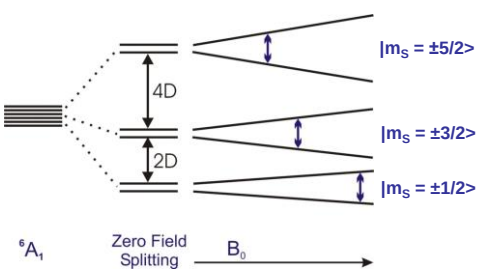
- These spacing are also called ***zero-field splittings***, or ZFS.

Energy Levels for S = 5/2 System



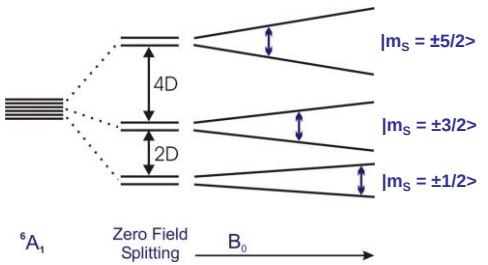
- The *S* = 5/2 multiplet forms three Kramers' doublets that are separated from the others by energies significantly larger than the ≈0.3-cm^{-1} microwave quantum (X-band).

Energy Levels for S = 5/2 System



- The degeneracy between the pairs is lifted in an external field.
- Since the ***zero field splitting*** is very large, the external field-induced splitting only allows for the occurrence of EPR transitions within each (split) pair of levels.
  ⇨ Only intra-doublet transitions observed in EPR.

Energy Levels for S = 5/2 System



- There is no crossing over and mixing of the energy levels.
- For Kramers' systems each Kramer pair can give rise to its own resonance.
- Each of these can be described in terms of an effective $S = ½$ spectrum with three ***effective g-values***.

Half-Integer / Kramers' Systems

$$h\nu = g^{eff}\beta B$$

- g^{eff} encompasses the real *g*-values plus the effect of the ***zero-field interaction***.
- Just like the *g*-value and *A*-values also the zero-field interaction parameter can be anisotropic and have three values D_x, D_y, and D_z.

Half-Integer / Kramers' Systems

$$h\nu = g^{eff}\beta B$$

- In contrast to *g* and *A*, however, the three D_i's are not independent because $D_x^2 + D_y^2 + D_z^2 = 0$, and so they can be reduced to two independent parameters by redefinition:

$$D = \frac{3D_z}{2} \text{ and } E = \frac{D_x - D_y}{2}$$

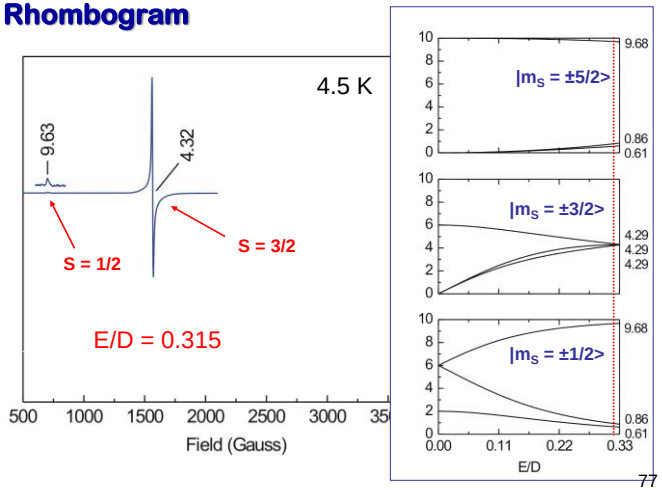
- We can also define a rhombicity

$$\eta = {}^{E}/_{D} \text{ with } 0 \le \eta \le {}^{1}/_{3}$$

Half-Integer / Kramers' Systems

- From the complete energy matrix it can be derived that under the so-called ***weak-field limit (Zeeman interaction << zero-field interaction)*** the three elements of the real *g*-tensor, g_x, g_y, and g_z, can be fixed at 2.00 and that the shape of the EPR spectra, the effective *g*-values, is a function of the rhombicity *E/D*.
- The relationship of the effective *g*-values versus the rhombicity can be plotted in two-dimensional graphs, so-called ***rhombograms***.

Rhombogram



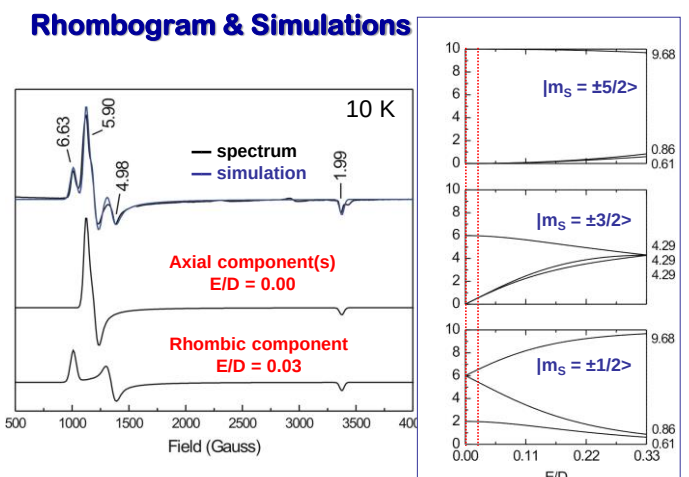
77

Mn^{2+}

- The somewhat simplified description of the EPR spectra of the different Fe^{3+} systems and iron-sulfur-cluster containing proteins was possible due to the fact that they all fall within the weak-field limit (Zeeman interaction << zero-field interaction).
- It is also possible that the zero-field interaction is much weaker than the Zeeman interaction, and this "strong-field limit" hold for six-coordinate Mn^{2+}, which is not only biologically relevant as a site in some manganese proteins, but also because this is a very common contaminant of biological preparations.

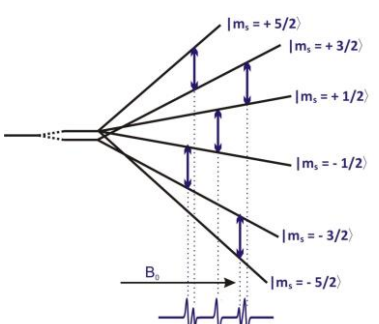
79

Rhombogram & Simulations



78

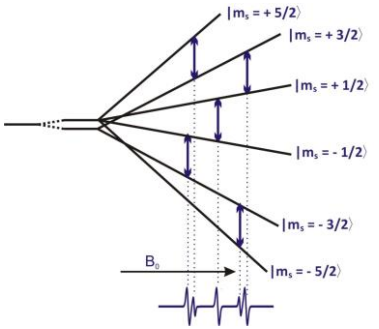
Mn^{2+}



- The electronic spin state of Mn^{2+} is S = 5/2.
- Six energy states with the electron spin magnetic quantum number, m_s = 5/2, 3/2, 1/2. -1/2, -3/2, and -5/2 arise due to the Zeeman effect.

80

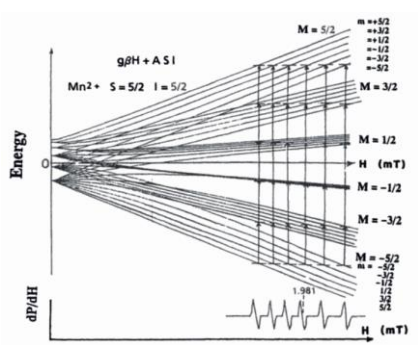
Mn^{2+}



- Pure cubic (e.g., octahedral) situation: D and E are zero, g is isotropic, because of the high spin a new zero-field energy term exists that produces EPR anisotropy.
- The zero-field splitting for B‖z are shown in the Figure.

81

Mn^{2+}

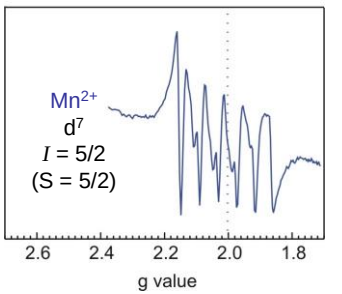


- Due to the nuclear spin magnetic quantum number, all lines will be further split into six hyperfine lines, m = 5/2, 3/2, 1/2. -1/2, -3/2, and -5/2.
- Note that due to second-order effects the energy level splitting by the Zeeman effect is not linear.

82

Mn^{2+}

- The energy level diagram predicts that the spectrum is dominated by the m_s = +1/2 ↔ -1/2 transition and shows the presence of six hyperfine lines each split by a small anisotropy induced by the zero-field splitting.



- In between the six hyperfine lines there are five pairs of weak lines from forbidden $\Delta m_I = \pm 1$ transitions with an order of magnitude lower intensity than the main lines.
- This whole ms = ±1/2 spectrum is on top of a very broad, rather structureless feature that is the sum of all the other five Δm_s =1 transitions (e.g., m_s = -3/2 ← -5/2).

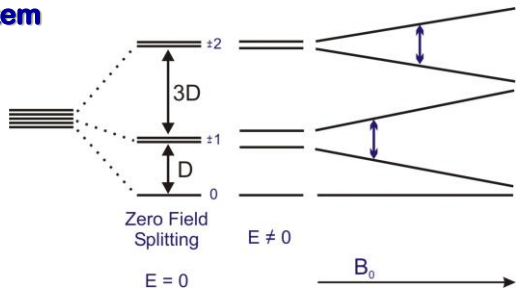
83

Integer / non-Kramers' Systems

- Non-Kramers' systems or integer systems are systems with S = 1, 2, 3, 4.
- These systems are very seldom observed in biological systems.
- One of the reasons is that just as in the Kramers' systems the energy levels are organized in doublets (and one singlet, |0⟩).
- These doublets, however, are split even at zero field and this splitting is generally greater than the energy of the X-band radiation.
- This means that in most cases the signals cannot be detected.

84

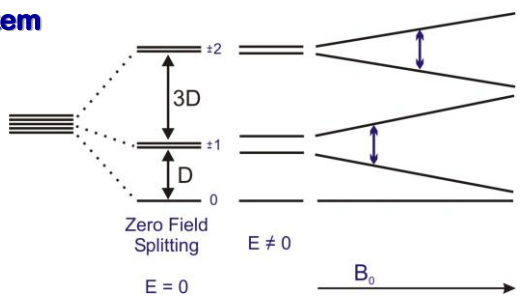
S = 2 System



- For $E = 0$, the effective g-values are $g_{xyz} = 0, 0, 4$ (for $|\pm1\rangle$ doublet) and $g_{xyz} = 0, 0, 8$ (for $|\pm2\rangle$ doublet), purely axial.
- This means that the signal are not detectable

85

S = 2 System



- For $E \neq 0$, the signals become more rhombic and mixing of the wave function takes place
- Signal can be detected in perpendicular-mode EPR: $|\Delta m_s| = 1$ or in parallel-mode EPR $|\Delta m_s| = 0$
- Since the levels are already split at $B_0 = 0$ the peaks will be shifted to higher g-values.

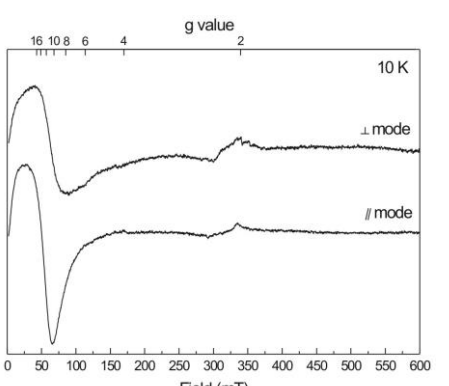
86

S = 2 System

- Due to several mechanisms the EPR signals are very broad and deformed and not much information can be obtained from the signals itself.
- For most systems however this zero-filed splitting is larger than the microwave energy at X-band and no signal will be detected.

87

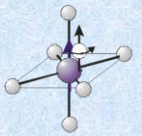
S = 2 System



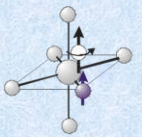
- Example of the spectra detected for the $S = 2$ $[3Fe\text{-}4S]^0$ cluster from hydrogenase from *Allochromatium vinosum*.

88

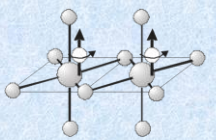
Hyperfine Interactions



Hyperfine interaction
Interaction of the electron spin with the nuclear spin of the metal ion nucleus



Super hyperfine interaction
Interaction of the electron spin with first coordinate sphere ligands nuclei



Spin-spin interaction
Interaction of the electron spin with other electron spins within 10 Å distance.

89

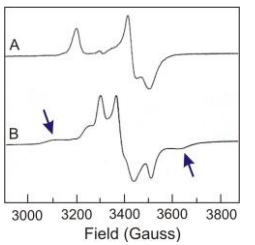
Spin-Spin Interaction

- In principle one could expect to see two signals for each paramagnetic species present and both signal would be split due to the spin-spin interaction.
- The distance of the split peaks would be dependent on the distance between the two species in the molecule
- This would only happen when the *g*-tensors of both species are linear.
- When the *g*-tensors are not parallel, however, the spectra will change significantly.

90

Spin-Spin Interaction

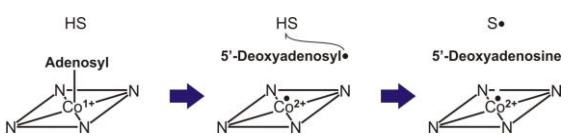
A) $[4Fe\text{-}4S]^{+}$ signal detected in a ferredoxin from *Bacillus stearothermophilus*.
B) Signal detected in a so-called 8Fe ferredoxin from *Clostridium pasteurianum*. In this sample two $[4Fe\text{-}4S]^{+}$ clusters are present.



Spectrum B does not look like two overlapping signals. A more complex signal is now detected. The broad wings in the EPR spectrum (indicated by the arrows) are typically found for two interacting clusters.

91

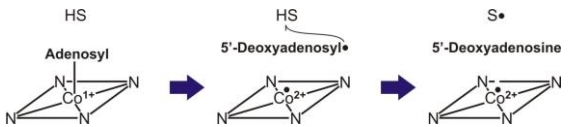
Spin-Spin Interaction



- Adenosylcobalamin (coenzyme B_{12})-dependent isomerases.
- Catalyze skeletal rearrangements via a radical mechanism.
- The reaction starts with the generation of the 5'-deoxyadenosyl radical and cob(II)alamin from enzyme-bound adenosylcobalamin by homolysis of the coenzyme's cobalt-carbon σ-bond in the presence of a substrate molecule SH.

92

Spin-Spin Interaction

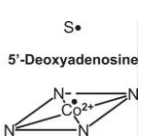


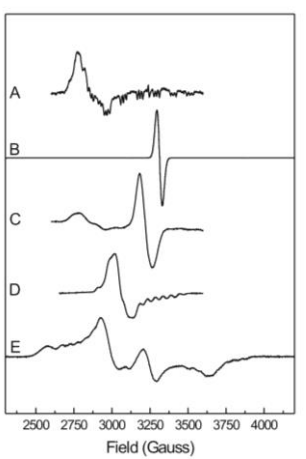
- Stereospecific hydrogen abstraction from the substrate molecule by the 5'-deoxyadenosyl radical gives 5'-deoxyadenosine and a substrate radical S•.
- At this point two paramagnetic species are present in the enzyme, the Co^{2+} species and the S• radical species.

93

Spin-Spin Interaction

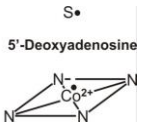
A) EPR spectrum of the Co^{2+} species by itself (g_{av} ≈2.18)
B) EPR of the radical species by itself (g = 2.0023)
C-E) The actual observed EPR spectra for different types of isomerases.





94

Spin-Spin Interaction

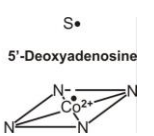


Different types of interactions dependent on the distance between the two interacting paramagnets.

- ***Through-space dipole-dipole interaction***: anisotropic interaction that follows a $1/r^3$ dependence on the spacing between the interacting centers
- ***Exchange interaction*** that depends on orbital overlap and spin polarization effects: isotropic interaction, which falls off approximately exponentially with the distance between the partners.

95

Weakly Coupled Spin Systems

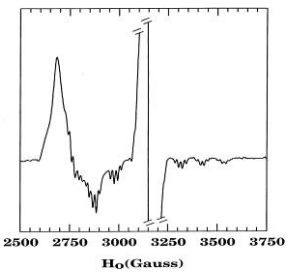


- ***At distances greater than approximately 9 Å, the exchange interaction creates a doublet splitting in the EPR spectrum of each partner***
- At closer distances, the exchange interaction mixes the two spin systems, such that their *g*-values become averaged and eventually converge to a triplet state at interspin separations of <7 Å.

96

Weakly Coupled Spin Systems

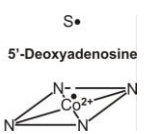
- EPR spectrum of hydroxyethylhydrazine-inactivated ethanolamine ammonia-lyase showing the presence of features corresponding to B_{12r} and a companion radical species with absorption near g = 2.0.



- The signals from the low-spin Co^{2+} and the partner radical were split by a combination of exchange and dipole-dipole coupling. This can be detected as the additional hyperfine splitting of the Co^{2+} signal.
- The amplitude of the signal of the radical centered at g = 2.0 is off scale.

97

Strongly Coupled Spin Systems



- At distances greater than approximately 9 Å, the exchange interaction creates a doublet splitting in the EPR spectrum of each partner

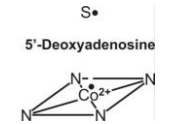
- ***At closer distances, the exchange interaction mixes the two spin systems, such that their g-values become averaged and eventually converge to a triplet state at interspin separations of <7 Å.***

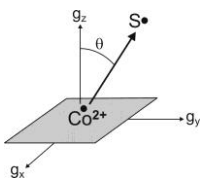
98

Strongly Coupled Spin Systems

Simulation of signals:

- For distances larger than 4-5 Å both paramagnets can be considered point dipoles.
- The zero-field splitting is described as a traceless tensor with an axial, *D*, and a rhombic, *E*, term. In the commonly used point-dipole approximation, $E \equiv 0$.
- The principal axis of the zero-field splitting normally contains the interspin vector. In simulations, Euler rotations (θ) are required to relate the axis system of the zero-field splitting tensor to a reference system, such as the *g*-axis of Co^{2+}.



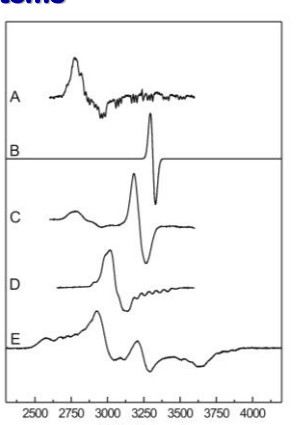


99

Strongly Coupled Spin Systems

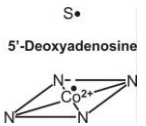
(C) Signal of the coupled Co^{2+}/radical species in ethanolamine ammonia lyase after reacting with ethanolamine. The interspin distance is 8.7 Å and θ = 25°

(D) Coupled Co^{2+}/radical species in lysine-5,6-aminomutase after reacting with 4-thialysine. The interspin distance is 7.0 Å and θ = 43°.



100

Strongly Coupled Spin Systems



- When there is a strong coupling between the cobalt and the radical species, the EPR spectra becomes a hybrid of both the cobalt and the radical EPR signals and exhibit an average *g*-value of ≈ 2.1 that arises from coupling between a carbon centered radical (g = 2.0023) with cob(II)alamin (g_{av} ≈ 2.18).
- The signals are due to a 'hybrid' triplet spin system comprising both paramagnets.

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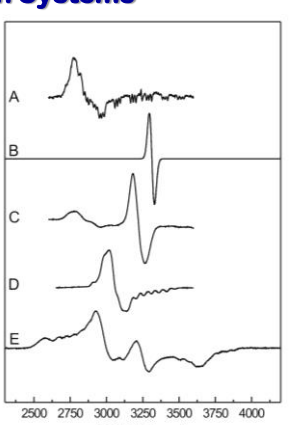
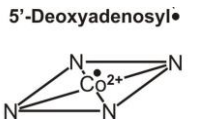
Very Strongly Coupled Spin Systems

(E) Coupled Co^{2+}/radical species in diol dehydratase after reacting with 5'-deoxy-3',4'-anhydroadenosylcobalamin. The interspin distance is 3.5 Å and θ = 75°.



102

Very Strongly Coupled Spin Systems



- When there is a very strong coupling the EPR spectrum does not resemble that of Co^{2+} or a radical species.
- However, it is still consistent with a rhombic triplet-state species. A prominent half-field transition at around g = 4 can be detected (not shown).
- Note that the EPR spectrum covers a wide area.
- The close spacing of the unpaired electrons, together with the spin delocalization within the allylic radical, requires a higher level of treatment than the point-dipole approximation.

103

g-Strain

- We know from folding studies and from structural NMR and X-ray studies that samples of proteins come with a distribution of conformations.
- For EPR this means that the paramagnet in each molecule has a slightly different structural surrounding and thus a slightly different *g*-value.
- This structural inhomogeneity or ***g-strain*** is reflected in the spectroscopy in the form of an inhomogeneous line shape.
- This normally results in a change from a ***Lorentzian*** to ***Gaussian*** line shape. An important consequence of this *g*-value anisotropy is that the line width, *W*, is in general, also isotropic.

104

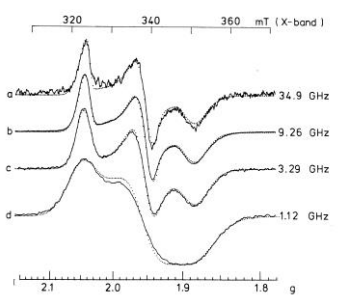
g-Strain

- Most of the time we do not have to worry about this, but particularly in the EPR spectra of the iron-sulfur clusters *g*-strain can have a big effect on the shape of the EPR spectrum and therefore on the simulation and interpretation of the EPR data.

105

g-Strain

- The most noticeable difference is now that the linewidth, plotted on a *g*-scale does not change when the spectra are measured at higher frequency.



- This effect is shown in the figure for the [4Fe-4S] cluster detected in spinach-leaf ferredoxin. The line width is very similar in the range of 35 to 3.3 GHz.
- At 1.1 GHz a broadening is detected due to unresolved hyperfine coupling.

106

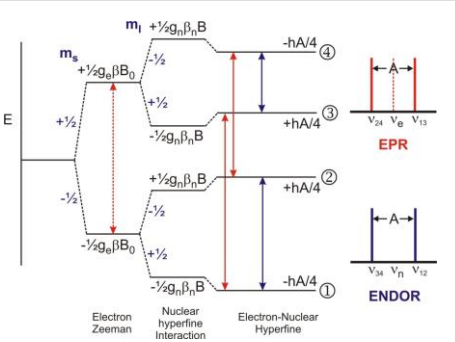
ENDOR

- Nuclear hyperfine splitting might not always be resolved but might be hidden in the EPR peaks.
- Techniques have been developed to detect these interactions: ***E***lectron ***S***pin ***E***cho ***E***nvelope ***M***odulation (ESEEM) and ***E***lectron-***N***uclear ***D***ouble ***R***esonance (ENDOR) spectroscopies.
- In transition metal complexes and metalloproteins, magnetic nuclei such as ^{1}H, ^{2}H, ^{13}C, ^{14}N, ^{15}N, ^{17}O, ^{31}P and ^{33}S, in the vicinity (2-12 Å) of the paramagnetic metal ion can be detected by these techniques.
- Identification of the presence of a particular ligand nucleus, and under favorable circumstances metal-ligand nuclei distances and angles can be obtained.

107

ENDOR

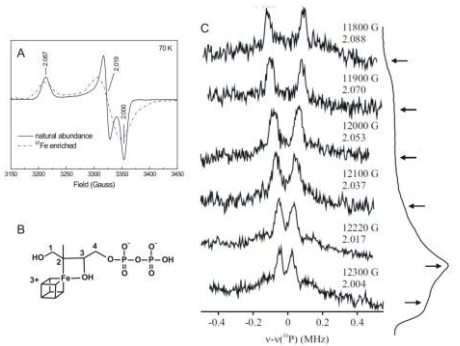
- Energy level diagram for an $S = ½$, $I = ½$ spin system.
- The *g*-value and hyperfine coupling are assumed to be isotropic.



- The red lines show the allowed EPR transitions and the stick EPR spectrum.
- The blue lines show the NMR (ENDOR) transitions and the stick ENDOR spectrum.

108

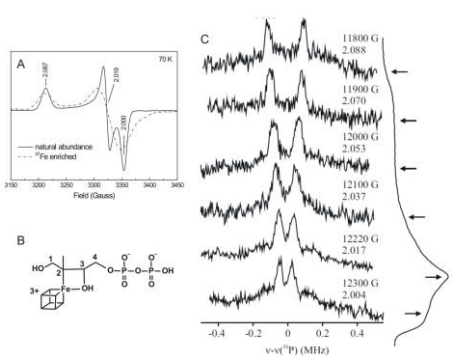
ENDOR



A) EPR spectrum obtained for IspG upon incubation with the substrate MEcPP and the reductant dithionite

B) Proposed structure for the reaction intermediate

ENDOR



C) ^{31}P-ENDOR spectra. Spectra were collected at the fields and *g*-values indicated, and are shown alongside the respective pulse-echo detected EPR spectra.
A cluster-^{31}P distance of 6.6 Å was calculated

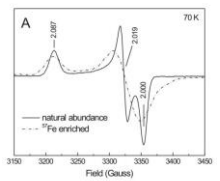
2D-HYSCORE

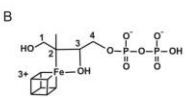
- ***E***lectron **S**pin ***E***cho ***E***nvelope ***M***odulation (ESEEM) is an important technique for measuring the hyperfine interaction parameters between electron spins and nearby nuclear spins.
- From the analysis of the ESEEM signals detailed information about electron spin density distribution, distances and bonding angles is gained.
- An extension of this technique is ***HY***perfine ***S***ub-level ***COR***r***E***lation (2D-HYSCORE). This technique is essentially a two dimensional ESEEM experiment in which correlation is transferred from one electron spin manifold to another.

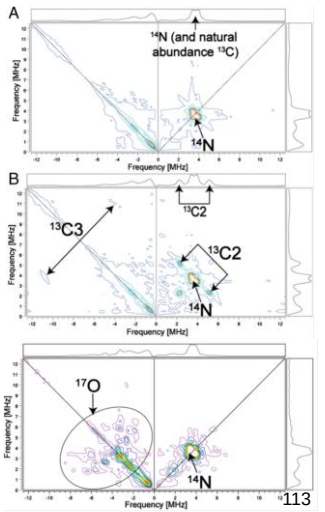
2D-HYSCORE

- HYSCORE allows one to take a complicated ESEEM spectrum and extend the data into a second dimension. Peaks appearing in the upper right and lower left quadrants of the 2D spectra typically arise from nuclei in which the hyperfine coupling is less than the Larmor frequency. They appear at the Larmor frequency, separated by the hyperfine coupling. Peaks from nuclei in which the hyperfine interaction is greater than the Larmor frequency appear in the upper left and lower right quadrants of the spectra. Even with the complexity of the spectra, HYSCORE on systems with multiple nuclei can make ESEEM spectra that would be difficult or impossible to interpret much more manageable.

2D-HYSCORE







^{13}C and ^{17}O HYSCORE Spectra of the FeS_A species in IspG

Quantum Mechanical Description

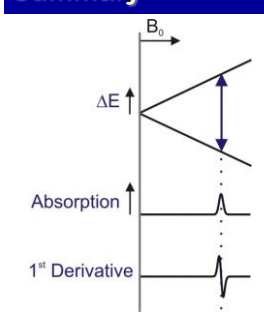
- Simplified Schrödinger wave equation

$$H_s\psi_s = E\psi_s$$

$$H_s = \underbrace{\beta B \bullet g \bullet S}_{\text{Zeeman interaction}} + \underbrace{S \bullet A \bullet I}_{\text{Hyperfine interaction}} + \underbrace{S \bullet D \bullet S}_{\text{Zero field splitting or spin-spin interaction}} + \ldots\ldots\ldots$$

- Can sometimes be solved under a set of ***assumptions***
- Solutions sometimes analytical, sometimes need for numerical approach
- Cannot allows be solved

Summary



Resonance condition:

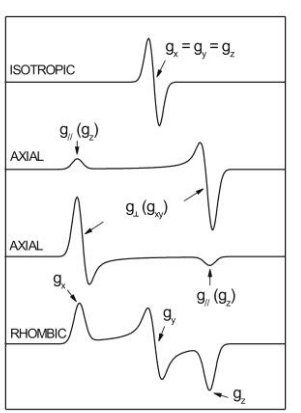
$$\Delta E = h\nu = g_e\beta B_0$$

For X-band:

$$g = 0.7145\frac{\nu\,(MHz)}{B\,(Gauss)}$$

Line Shape of EPR Spectra for $S = ½$ Systems

- Four basic shapes.
- When more than three peaks are detected the signal could be split due to spin interaction or there could be more than one signal present.



Line Shape of EPR Spectra for High-Spin Systems

- Half-integer/non-Kramers:

  S = 3/2, 5/2, 7/2, 9/2

- Rhombograms will help with the identification of the spin state, determination of which spin doublets are detectable and determination of the E/D value.

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Practical Aspects of EPR Spectrometry

1) Metal-Ion Type Identification
2) Optimal Measuring Conditions (*T*,*P*)
3) The X-band EPR Spectrometer
4) Spectrometer Parameters
5) Spin Intensity
6) Redox Titrations
7) Freeze-Quench Experiments
8) Simulation of EPR Spectra
9) EPR on Whole Cells/Cell Extract
10) Visual Rhombo

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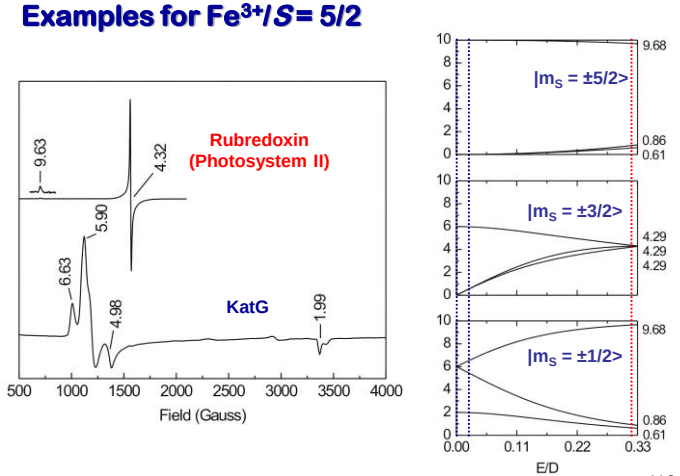
Examples for Fe^{3+}/*S* = 5/2



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1) Metal-Ion Type Identification

- Which redox state is EPR active?

Metal Ion	Electron Configuration	Spin State
Fe^{2+}	d^6	S = 0 (ls) or S = 2 (hs)
Fe^{3+}	d^5	S = 5/2 (hs)
Ni^{1+}	d^9	S = ½
Ni^{2+}	d^8	S = 0 or S = 1
Ni^{3+}	d^7	S = ½
Cu^{1+}	d^{10}	S = 0
Cu^{2+}	d^9	S = ½

Prepare different samples: 1) as such
2) reduced (dithionite)
3) oxidized (ferricyanide)

- How many unpaired electrons? Different spin states!

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Metal-Ion Type Identification

- Has the metal a nuclear spin?

Atom	Isotope	Spin (abundance)
V	*50*, *51*	^{50}V, 6 (0.25); ^{51}V, $^{7}/_{2}$ (99.75)
Mn	*55*	$^{5}/_{2}$
Fe	54, 56, *57*, 58	$^{1}/_{2}$ (2.119)
Co	*59*	$^{7}/_{2}$
Ni	58, 60, *61*, 62	$^{3}/_{2}$ (1.14)
Cu	*63*, *65*	^{63}Cu, $^{3}/_{2}$ (69.17); ^{65}Cu, $^{3}/_{2}$ (30.83)
Mo	92, 94, *95*, 96, *97*, 98, 100	^{95}Mo, $^{5}/_{2}$ (15.92); ^{97}Mo, $^{5}/_{2}$ (9.55)
W	180, 182, *183*, 184, 186	$^{1}/_{2}$ (14.3)

Is the signal going to be split into 2 I + 1 lines?

- In general: The spin–orbit coupling parameter is positive for systems with less than half filled outer shells and negative for those with more than half filled shells, which means that the former have $g<g_e$ and the latter have $g>g_e$.

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2) Optimal measuring Conditions (T, P)

- There is a need to measure at lower temperatures!
- EPR frequencies (1-100 GHz) are in the microwave range!
- Aqueous solutions will warm up in the EPR cavity at RT! This effect is absent in frozen samples.

Do-it-yourself microwave source

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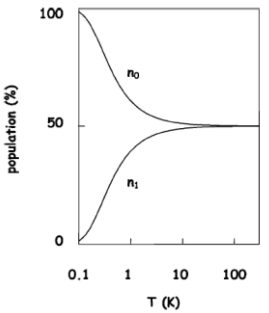
The Need for Lower Temperatures

The energy difference between the two energy level due to the Zeeman splitting is very small, ~0.3 cm^{-1} for X-band EPR.

Based on the Boltzmann distribution

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



it can be shown that only at low temperatures there will be enough difference in the population of the S = -½ level (n_0) and the S = ½ level (n_1) to create a signal.

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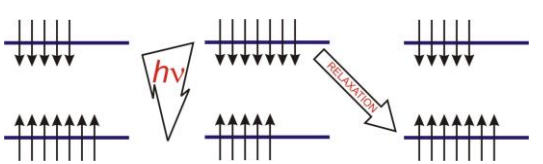
Spin-Lattice Relaxation



EPR on metalloproteins:

- the relaxation rate *de*creases with *de*creasing temperature; and
- the relaxation rate is anisotropic (*i.e.* is different for different parts of the spectrum).

When too much power is applied the signal will saturate:
Power saturation!

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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-*in*dependent process: *in*homogeneous 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.

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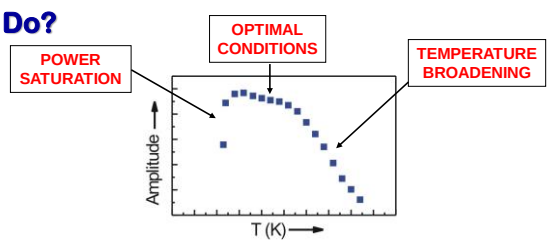
What to Do?



- Optimal measuring conditions (*T*,*P*) are determined by the interplay of the Boltzmann distribution, the Heisenberg uncertainty relation, the spin–lattice relaxation rate, and the conformational distribution of molecular structure.
- How do I find the correct measuring condition?
  1) ***Make a Curie Plot***
  2) ***Make Power Plots***

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Power Plots

- The power in EPR is expressed in ***decibels (dB)*** ***attenuation***
- X-band microwave sources have a constant output that is usually leveled off at 200 mW (= 0 dB):

$$P(\mathrm{dB}) = -10 \times \log(0.2/P(\mathrm{W}))$$

- logarithmic scale: every 10 dB attenuation means an order-of-magnitude reduction in power.
- A good X-band bridge operates at power levels between 0 and -60 dB

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Power Plots

Relationship between the amplitude, gain and the power in dB:

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

Both power and gain scales are logarithmic!

Need for low temperatures and high power, but this could lead to power saturation!

Practical rule: the amplitude of a *non*-saturated EPR signal does not change if a reduction in power by 1 dB is compensated by an increase in gain by one step.

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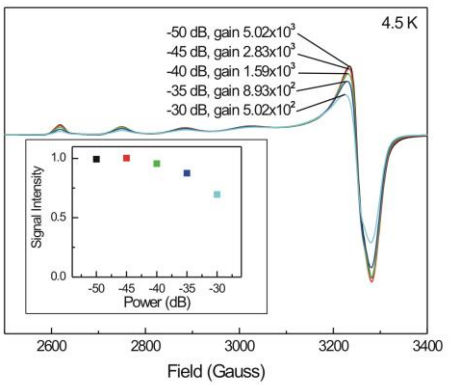
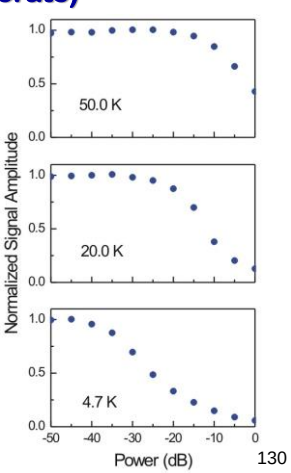
Power Plot (Copper Perchlorate)

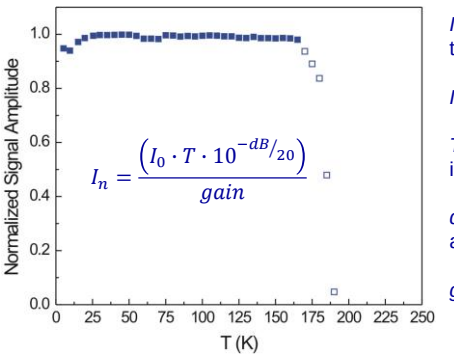


Power Plot (Copper Perchlorate)

- The relaxation rate ***in***creases with ***in***creasing temperature.
- Therefore if a signal does not saturate at a certain power at a certain temperature it will also not saturate at the same power at a higher temperature.
- The ***temperature behavior*** or ***Curie behavior*** will be different for different species.



Curie Plot (Copper Perchlorate)



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

I_n, normalized value for the intensity;

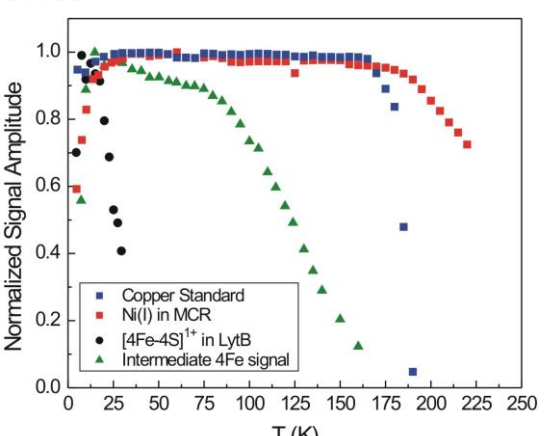
I_0, observed intensity;

T, absolute temperature in K;

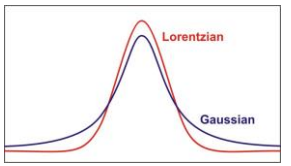
dB, reading of the attenuator;

$gain$, gain

Curie Plot



Line Shape



- The basic form of an EPR peak is described by the ***Lorentz distribution***. The ***Lorentzian line shape*** is also frequently called the ***homogeneous line shape***.
- In biological samples the paramagnet in each molecule has a slightly different structural surrounding and thus a slightly different *g*-value.
- This structural inhomogeneity is reflected in the form of an ***inhomogeneous line shape*** in addition to the Lorentzian shape.
- At low temperature the contribution from homogeneous broadening is small and the line shape can be described by the ***Gaussian distribution***.

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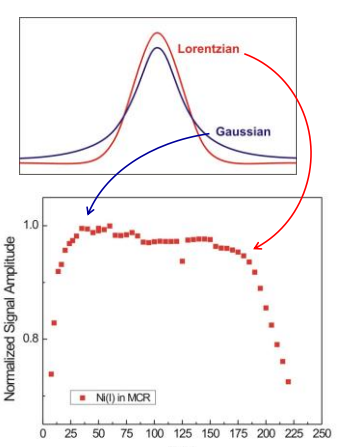
Line Shape

- At relatively high temperature a Lorentzian line shape will be observed, while a Gaussian line will be observed at relatively low temperatures
- The Gaussian shape will be broader.
- Preference to measure at the higher temperature end of Curie plot
- Practice better signal-to-noise at the lower end.



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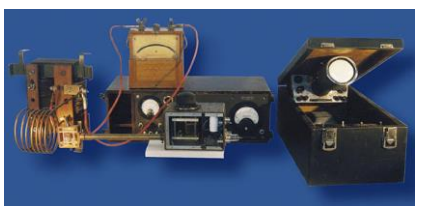
3) The X-band EPR Spectrometer

- In 1944, E.K. Zavoisky discovered magnetic resonance. Actually it was EPR on $CuCl_2$.



E.K. Zavoisky's first EPR system

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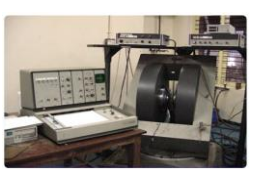
EPR Spectrometer

Varian E3



Varian E4

Bruker ElexSys X-band

Bruker ElexSys W-band

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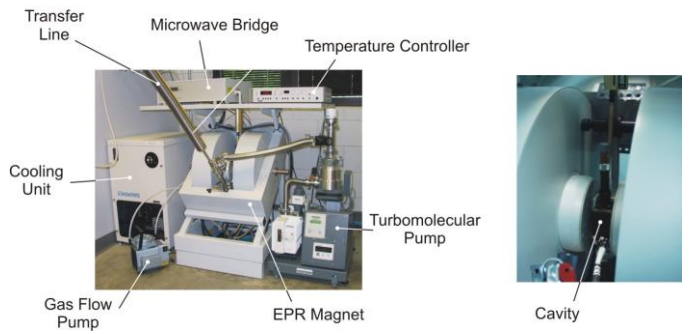
X-Band EPR Spectrometer



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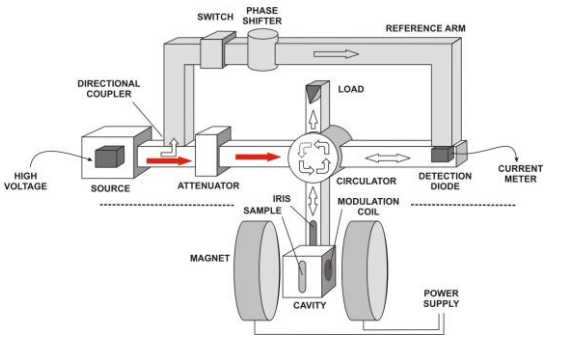

- On the left is a monochromatic source of microwaves of constant output (200 mW) and slightly (10%) tunable frequency.

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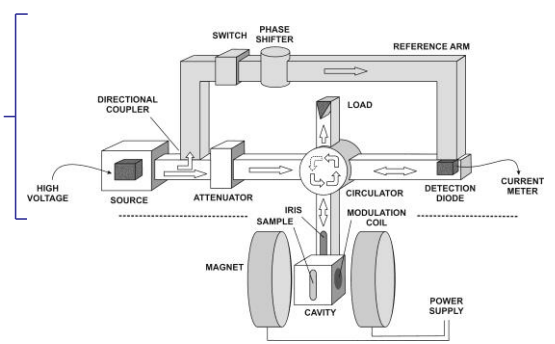
X-Band EPR Spectrometer

Microwave bridge



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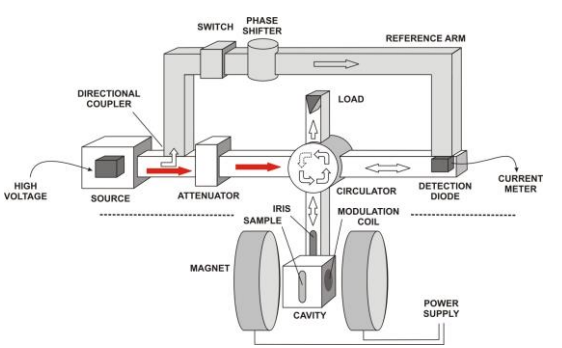

- The produced radiation is transferred by means of a rectangular, hollow wave guide to an attenuator where the 200 mW can be reduced by a factor between 1 and 10^6.

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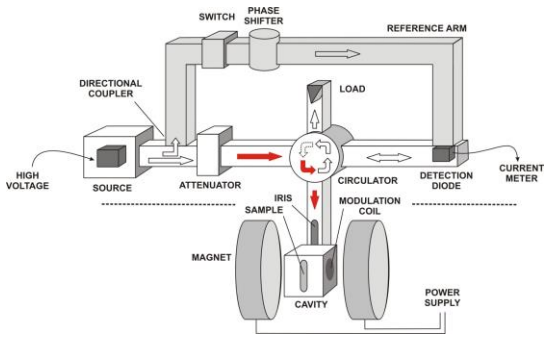

- The output of the attenuator is transferred with a waveguide to a circulator that forces the wave into the resonator/cavity.
- The entrance of the resonator is marked by the iris, a device to tune the amount of radiation reflected back out of the resonator.

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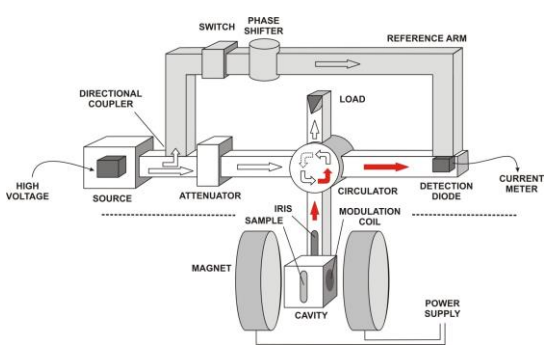

- The reflected radiation returns to the circulator and is directed to the diode for the detection of microwave intensity.

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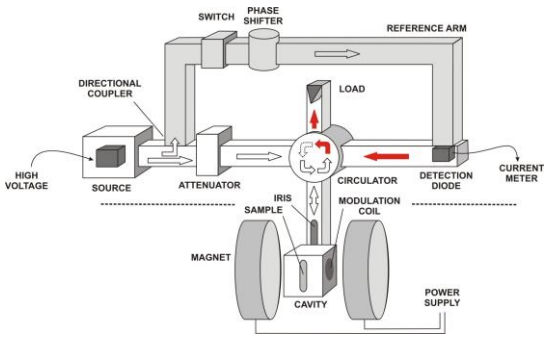

- Any remaining radiation that reflects back from the detector is forced by the circulator into the upward waveguide that ends in a wedge to convert the radiation into heat.

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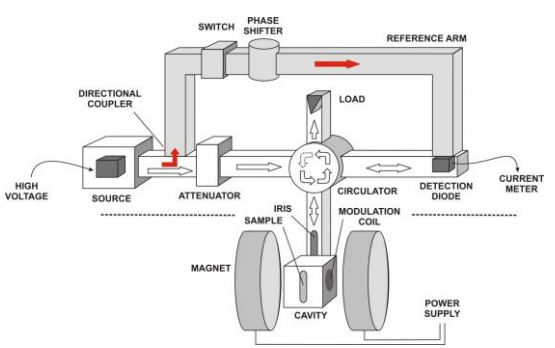

- A small amount of the 200 mW source output is directed through the reference arm directly to the detector to produce a constant working current.
- The reference arm contains a port that can be closed and a device to shift the phase of the wave.

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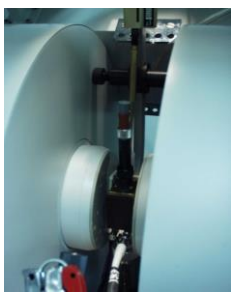
X-Band EPR Spectrometer

- Most EPR spectrometers are reflection spectrometers.
- 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.



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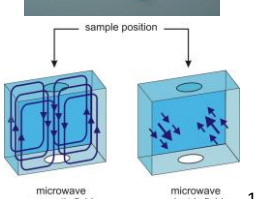
Cavity/EPR Resonator

- A microwave cavity is simply a metal box with a rectangular or cylindrical shape which resonates with microwaves much as an organ pipe resonates with sound waves.
- The resonator is designed to set up a pattern of standing microwaves in its interior.
- 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.



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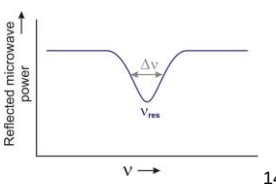
Cavity/EPR Resonator

- 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.



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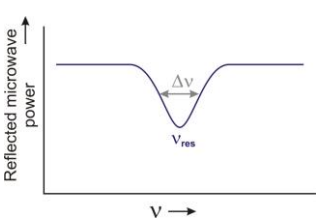
Cavity/EPR Resonator

- Cavities are characterized by their *Q* or quality factor, which indicates how efficiently the cavity stores microwave energy.



- We can measure *Q* factors easily:

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

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

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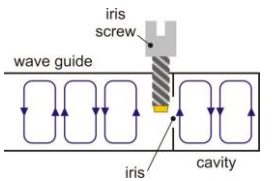
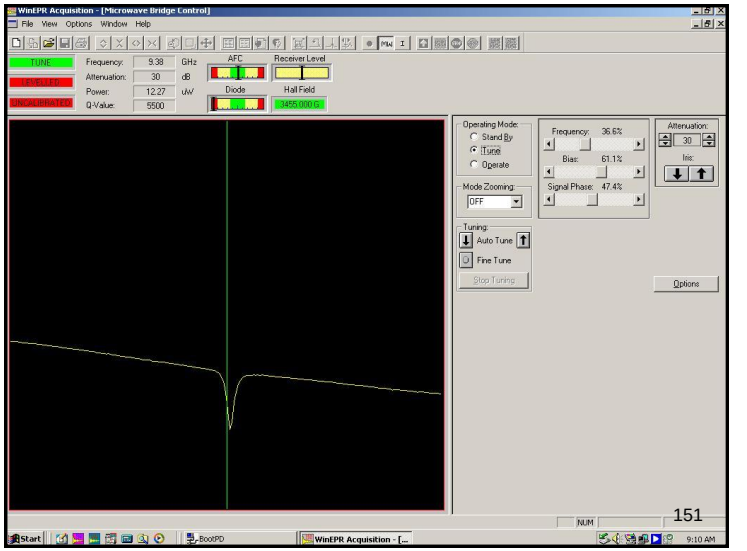
Cavity/EPR Resonator

- In order for the microwaves to enter the cavity one of its end walls must have an opening: ***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 waves enter the cavity, and no radiation is reflected out.



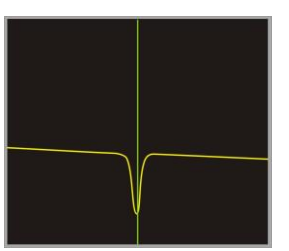


Cavity/EPR Resonator

- How do all of these properties of a cavity give rise to an EPR signal? 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.

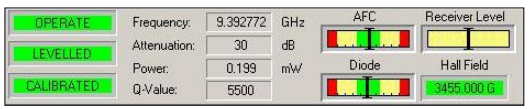
Tuning the Microwave Cavity and Bridge

- **Locate and center the "dip" on the display**.
- The pattern 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, the microwave source is set to oscillate at the same frequency as the cavity resonant frequency

Tuning the Microwave Cavity and Bridge



- **Tune the signal (reference) phase.** Adjust the Signal Phase until the depth of the dip is maximized and looks somewhat symmetric.
- **Adjust the bias level**. Adjust the Bias until the Diode meter needle is centered.
- **Critical coupling of the cavity**. Power is increased and the iris screw is adjusted to keep the diode current in the center.

153

4) Spectrometer Parameters

- ***Center Field*** and ***Sweep Width***

- For initial broad scans, a Sweep Width around 5000 Gauss is recommended. Set the Center Field value to 2600 Gauss. This means that the scan will start at 100 Gauss and stops at 5100 Gauss (*2500 Gauss below and 2500 Gauss above the Center Field value*).
- This scan will cover the complete area available with our magnet where signals might be detectable.

154

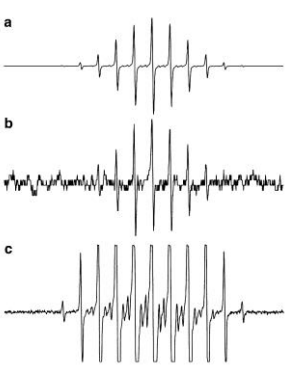
Spectrum Settings

- ***Points***
- Standard 1024 points. Can be increased to 4096 for wide scans to keep the resolution.
- It is advisable, however, to rescan the interesting parts of a wide scan.
- Subtractions are not possible if the amounts of points between the two spectra are different.

155

Spectrum Settings

- ***Gain***



- Use the full range of the digitizer (a), coincides with the screen display.
- If the receiver gain is too low the effect of digitization will be evident in the spectrum (b)
- At too high gain the signals will be clipped due to an overload in the signal channel (c).

156

Microwave Bridge Parameters

- ***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.

157

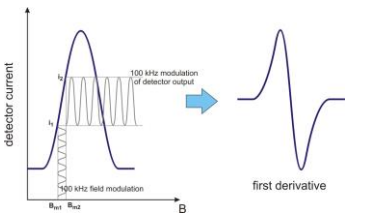
Phase Sensitive Detection

- Enhancement of the sensitivity of the spectrometer: less noise from the detection diode and the elimination of baseline instabilities due to the drift in DC electronics.
- The magnetic field at the site of the sample is modulated (varied) sinusoidally at the 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.
- Only the amplitude modulated signals are detected. Any signals which do not fulfill these requirements (i.e, noise and electrical interference) are suppressed.

158

Phase Sensitive Detection

- 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.

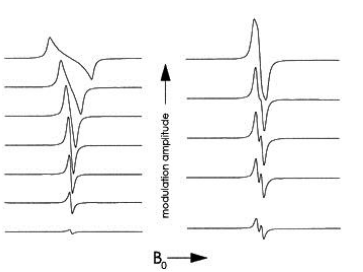


- As a result the first derivative of the signal is measured.
- Two new parameters: ***modulation amplitude***, and ***frequency***.

159

Field Modulation

- 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.



- 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. Also, if we use a modulation amplitude greater than the splitting between two EPR signals, we can no longer resolve the two signals.

160

Signal Channel Parameters

- ***Modulation frequency***: normally set to 100 kHz
- ***Modulation amplitude***: You can start with 6 Gauss. The larger this value the lower the value needed for the Receiver Gain, which means less noise. Excessive field modulation, however, broadens the EPR lines and does not contribute to a bigger signal. As a rule-of-thumb this value has to be smaller than the line width of your signal.

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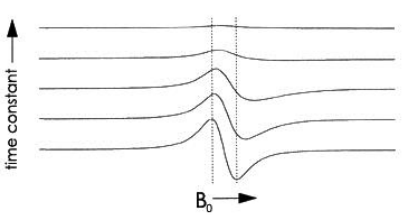
Time Constant



- 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, we can distort or even filter out the very signal which we are trying to extract from the noise. Also, the apparent field for resonance will shift.

162

Signal Channel Parameters

- ***Time Constant*** and ***Conversion Time***: 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.
- A longer Conversion Time, however, also improves the signal to noise ratio 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.
- With a sweep width off about **1000** Gauss a Conversion Time of **163.84** msec and a Time Constant of **163.84** msec can be used.

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Signal Averaging

- 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 $\sqrt{N}$, 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. Slow variations 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.

164

Spectrometer Parameters

- ***Center Field***, ***Sweep Width, Gain, Microwave power level***: sample dependent
- ***Modulation frequency***: normally set to 100 kHz
- ***Modulation amplitude***: normally set to 6 Gauss.
- ***Time Constant*** and ***Conversion Time***: same value! sweep width off **1000** Gauss; both **163.84** msec
- ***Number of X-Scans***: normally set to 1

Normalized Signal Intensity

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

where

I_n	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

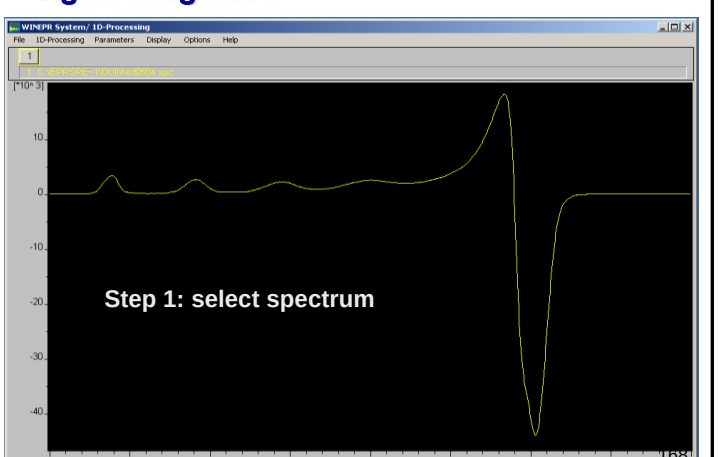
and

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

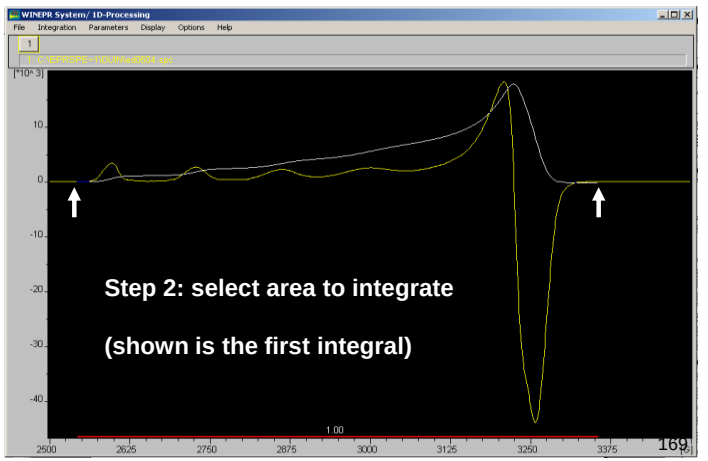
5) Spin Intensity

- Also known as ***spin counting***
- To calculate the amount of signal in a protein sample, the spin intensity can be compared with that of a standard with a known concentration (Copper perchlorate: 10 mM)
- Since an EPR spectrum is a first derivative, we have to integrate twice to obtain the intensity (I_0 = area under the absorption spectrum).
- In addition, corrections are needed for a number of parameters, to 'normalize' the spectra. Only then a direct comparison of double integral values of standard and unknown is possible:

Signal Integration



Signal Integration



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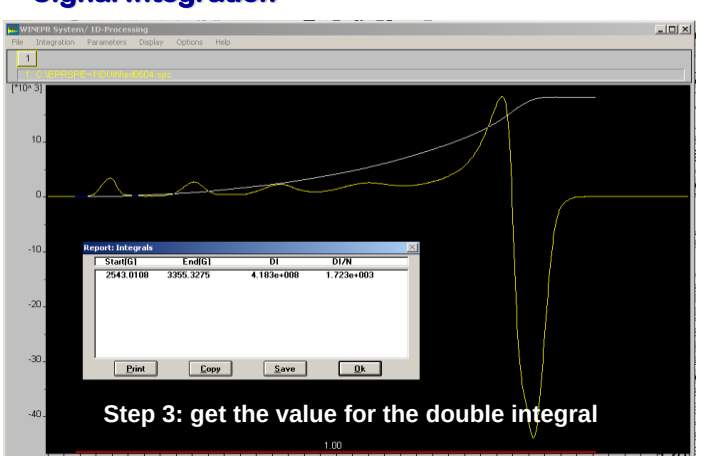
Signal Integration



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Comparison with 'Spin' Standard

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

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

- Keep measuring conditions the same: temperature, modulation amplitude, sweep time, amount of points, amount of scans (These are not averaged!)
- Measure samples on the same day!
- Correct for spin: $S(S+1)$

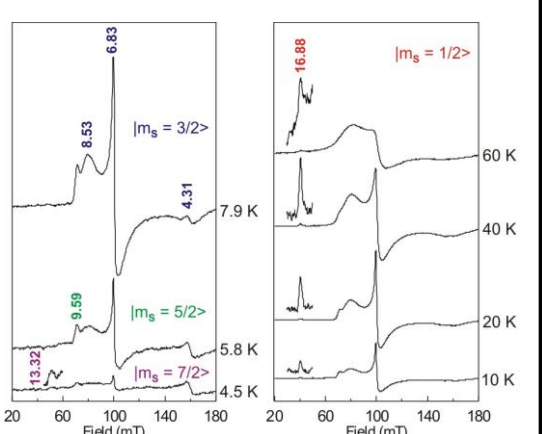
171

Signal Intensity???

Clostridium pasteurianum
$[2Fe\text{-}2S]^{2+}$

S = 9/2

(D < 0)



172

Signal Intensity???

- The effective spin-Hamiltonian suggests an easy way for quantification of high-spin spectra: one simply applies the double-integration procedure to the effective S_{eff} = 1/2 spectrum *as if* it were a real S = 1/2 spectrum, however, with a correction for the fractional population of the relevant doublet. (*Most of the time not possible!*)

- Exception: For *high spin ferric hemoproteins* ($D \approx +10$ cm^{-1}) in X-band at T = 4.2 K the fractional population of the $|m_S = \pm 1/2>$ doublet is very close to unity (0.999) therefore, quantification of the spectrum does not require a depopulation correction.

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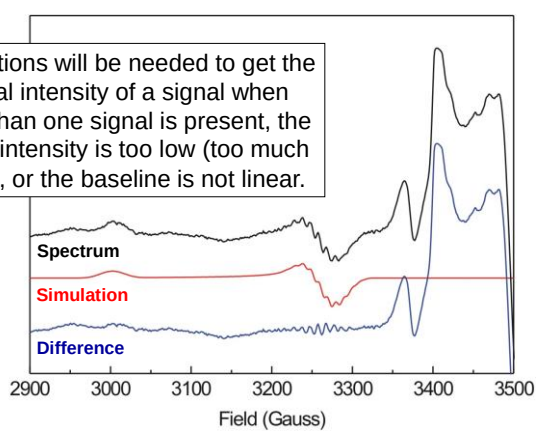
Signal Intensity ???

Simulations will be needed to get the signal intensity of a signal when more than one signal is present, the signal intensity is too low (too much noise), or the baseline is not linear.



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6) Redox Titrations

- With species that are only paramagnetic at a certain redox potential it is possible to do a redox titration and obtain the ***midpoint potential*** (E_m) of the redox couple.
- This is particular useful if you are studying proteins that are involved in electron transfer pathways.
- In these experiments the protein is titrated in both the oxidative direction with ferricyanide and in the reductive direction with dithionite. The potential can be measured with a combination Ag/AgCl electrode,
- A mixture of redox dyes is added to stabilize the redox potential outside the E_m region

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Redox Titrations

- When there is only a single paramagnetic species present the intensity of this signal can be determined directly.
- When more than one species are present you have to look for unique features, or the different components have to be simulated and their intensities determined.
- Plots of the intensity vs. the potential are generated.
- The points in the plot can be fitted with the ***Nernst equation:***

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

R (gas constant) = 8.314 J K^{-1} mol^{-1}; F (Faraday constant) = 9.649×10^4 C mol^{-1}; n is the number of moles of electrons

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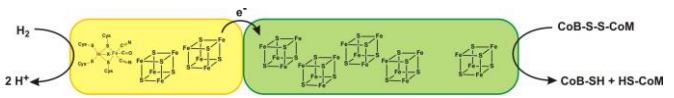
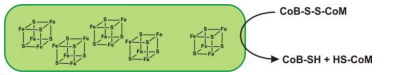
Redox Titrations

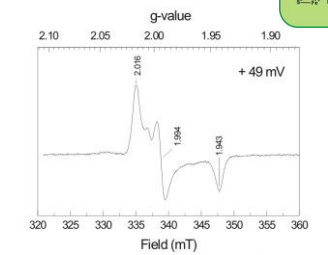
Heterodisulfide Reductase/Hydrogenase Complex:

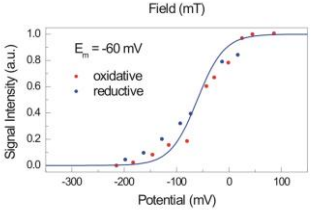


- Found in the cytosol of some Methanogens
- Electrons from hydrogen are used to break down the heterodisulfide CoB-S-S-CoM which allows the reuse of both cofactors.
- A proton gradient is generated in this process
- Except for one, all clusters are involved in electron transport.

Redox Titrations



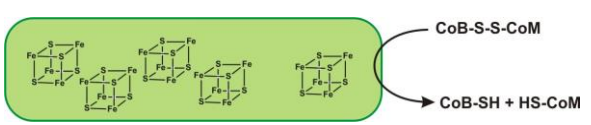


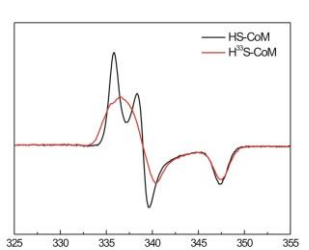


Cluster with bound HS-CoM behaves like a $[4Fe\text{-}4S]^{2+/3+}$ cluster (paramagnetic when oxidized)

E_m = -60 mV, pH 7.6, n = 1

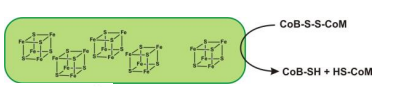
Redox Titrations

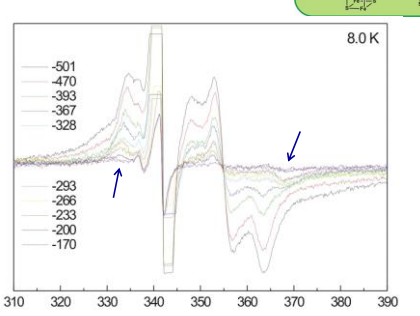




Labeling studies with $H^{33}S$-CoM (^{33}S: I = 3/2)

Redox Titrations

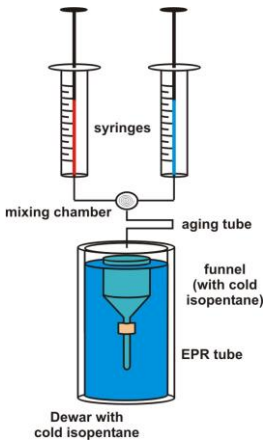




A single cluster gets reduced at relative high potential E_m = –153 mV, (see arrow)

More clusters get reduced at -293 mV and lower. The signal is more complex and broad due to spin coupling between the clusters

7) Freeze-Quench Experiments



- To follow a reaction involving paramagnetic species ***freeze-quench experiments*** can be performed.
- In this experiment enzyme is mixed with substrate and other compounds and EPR samples are made by rapid mixing and freezing.
- Multiple samples have to be made to get insight into the formation/disappearance of an EPR signal.

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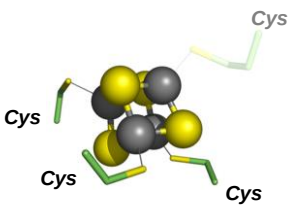
Role of IspG in Isoprene Synthesis

- IspG contains a single [4Fe-4S] cluster.
- The cluster is very unstable.
- Cluster falls apart when exposed to molecular oxygen.
- Instability probably caused by incomplete coordination.



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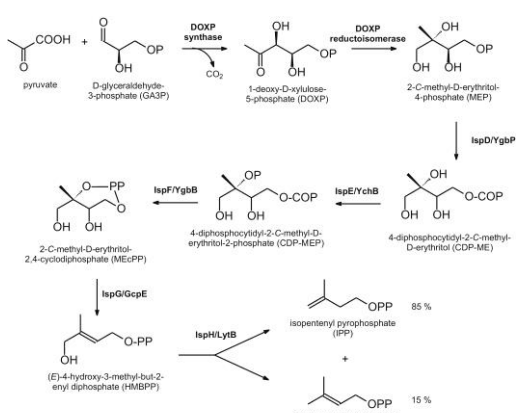
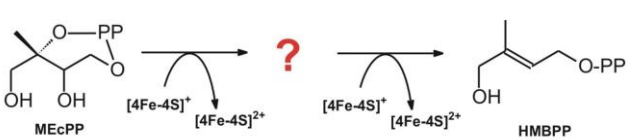
Role of IspG in Isoprene Synthesis



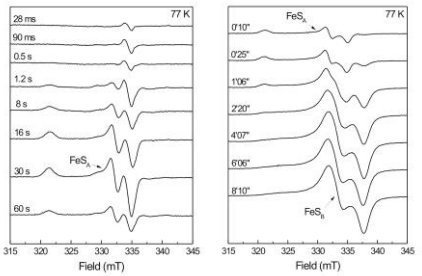
182

Role of IspG in Isoprene Synthesis



- The reaction is a reductive elimination of a hydroxyl group involving 2 electrons.
- A [4Fe-4S] cluster can only donate 1 electron at-a-time.
- Formation of radical species expected.

Freeze-Quench Experiments with IspG



- A transient isotropic signal is detected with maximal intensity at 90 ms.
- A transient rhombic signal, FeS_A, reaches maximal intensity at 30 s.
- A second rhombic signal, FeS_B, accumulates over time and reaches maximal intensity at 4 min.

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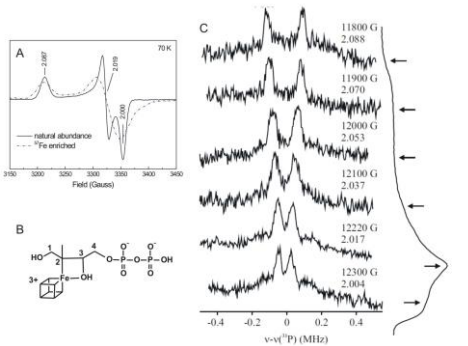
ENDOR



A) EPR spectrum obtained for IspG upon incubation with the substrate MEcPP and the reductant dithionite

B) Proposed structure for the reaction intermediate

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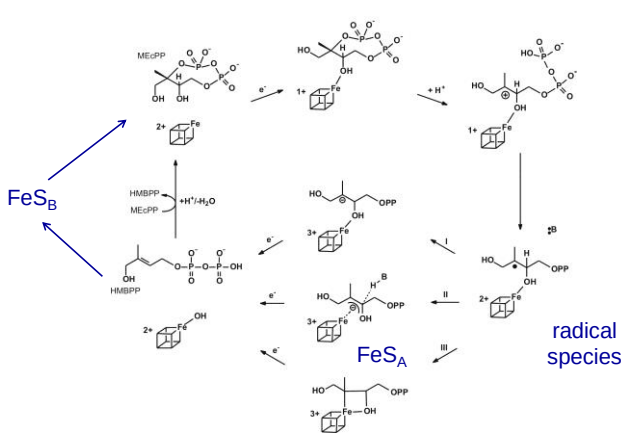
Freeze-Quench Experiments



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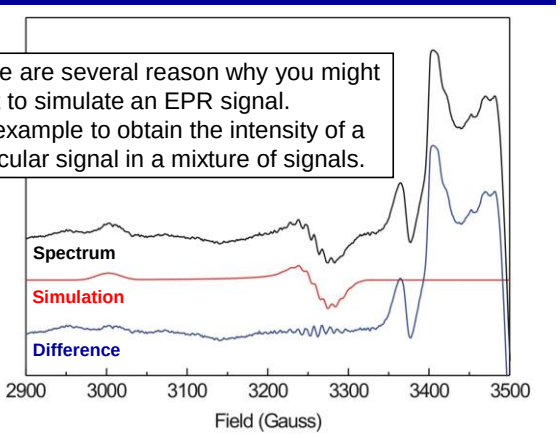
8) Simulation of EPR spectra

- There are several reason why you might want to simulate an EPR signal.
- For example to obtain the intensity of a particular signal in a mixture of signals.



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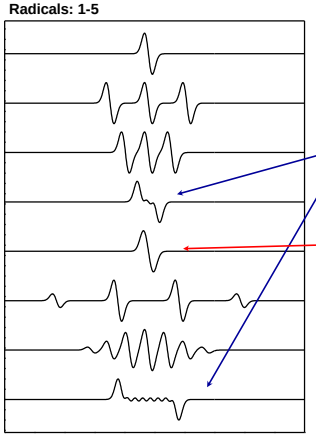
Simulations

Radicals: 1-5



Overlap between the peaks can cause a signal to look very weird

Unresolved hyperfine

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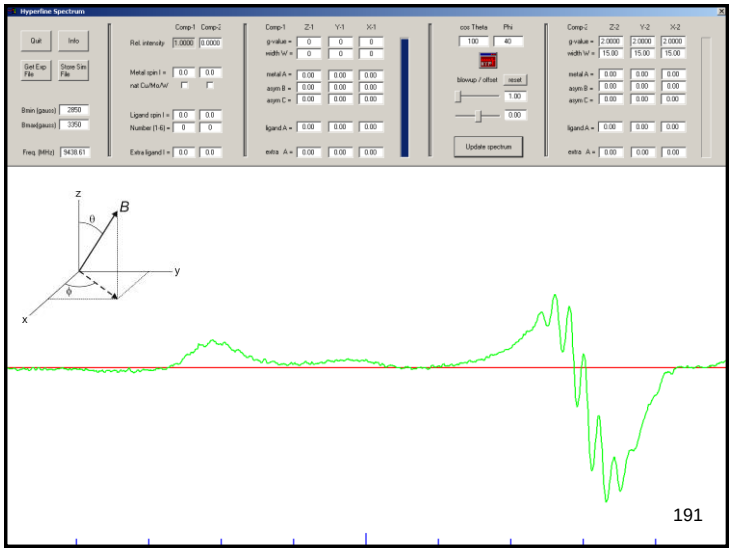

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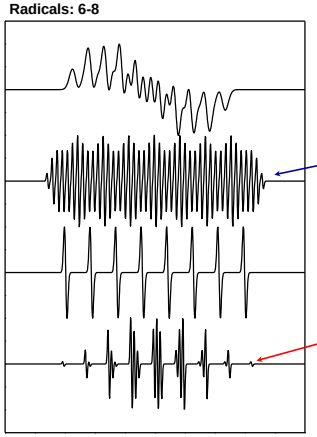
Simulations

Radicals: 6-8



Overlap between the hyperfine peaks makes it look like there are only 7 main peaks

A_N just a bit larger than A_H
$g_{iso} = 2.005$
$W = 0.7$
A_H (6x) = 14.8
A_N (1x) = 17.0

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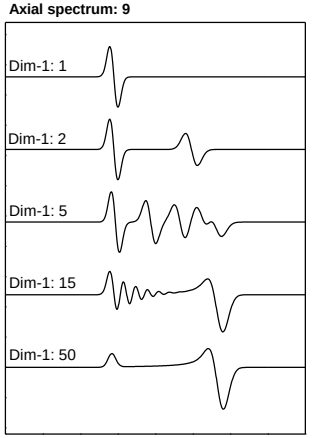
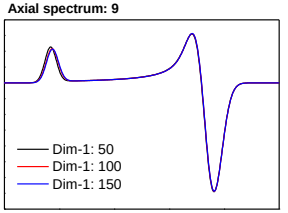
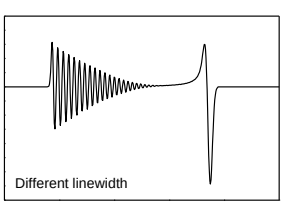
Simulations – Not Enough Orientations

Axial spectrum: 9



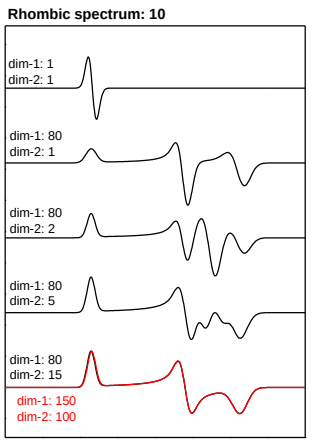
Axial spectrum: 9





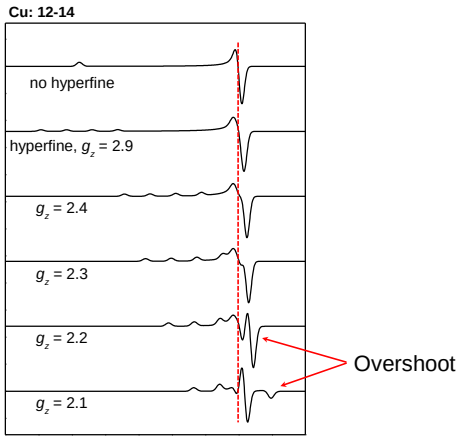
192

Simulations – Not Enough Orientations



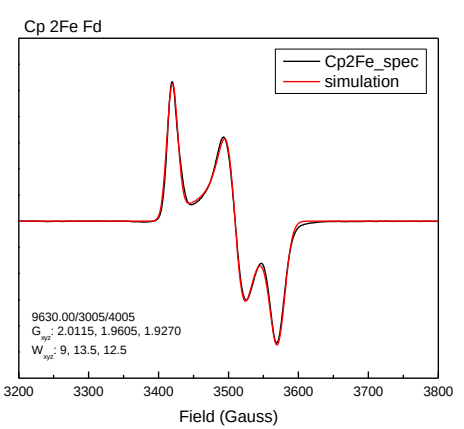
193

Simulations



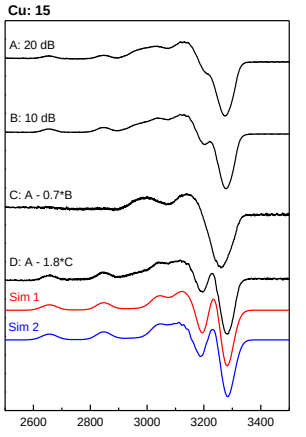
195

Simulations



194

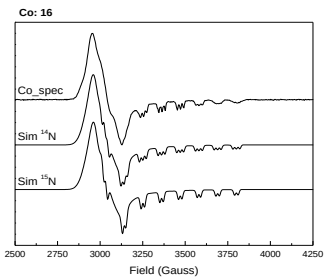
Simulations

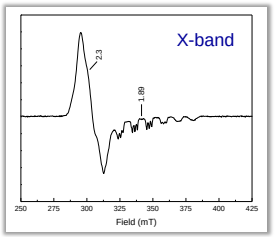


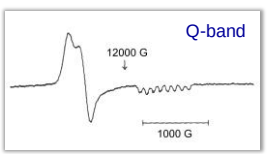
Sim 1	g_{xyz} =	2.2170, 2.0550, and 2.0550
	W_{xyz} =	30, 35, and 35
	A^{Cu}_{xyz} =	200, 20, and 20
Sim 2	g_{xyz} =	2.2170, 2.0550, and 2.0550
	W_{xyz} =	30, 9.5, and 9.5
	A^{Cu}_{xyz} =	200, 24, and 24
	A^{N}_{xyz} =	0, 15, and 15

196

Simulations



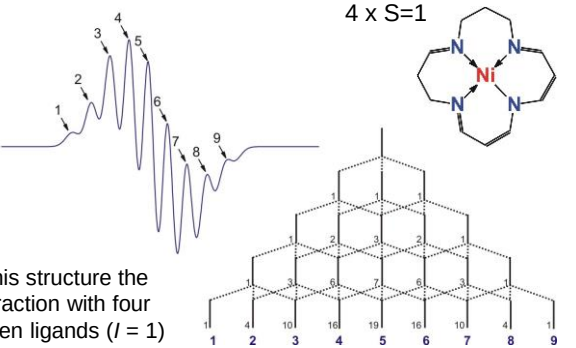




g_{xyz} = 2.275, 2.220, and 2.006
W_{xyz} = 25, 25, and 7.0
A^{Co}_{xyz} = 11, 11, and 111
A^{N}_{xyz} = 18, 18, and 18

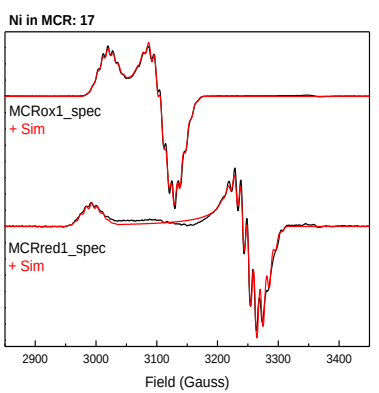
Hyperfine Interactions

4 x S=1

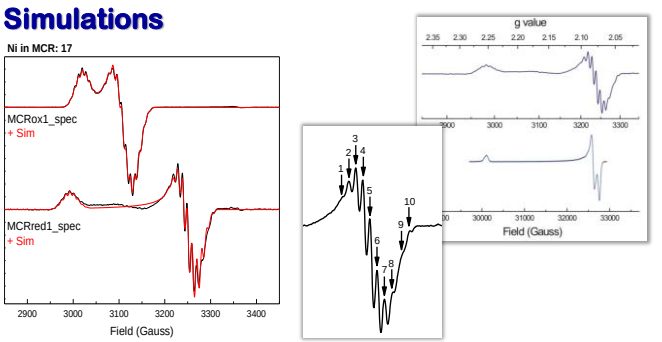


In this structure the interaction with four nitrogen ligands (I = 1) would result in 9 superhyperfine lines.

Simulations

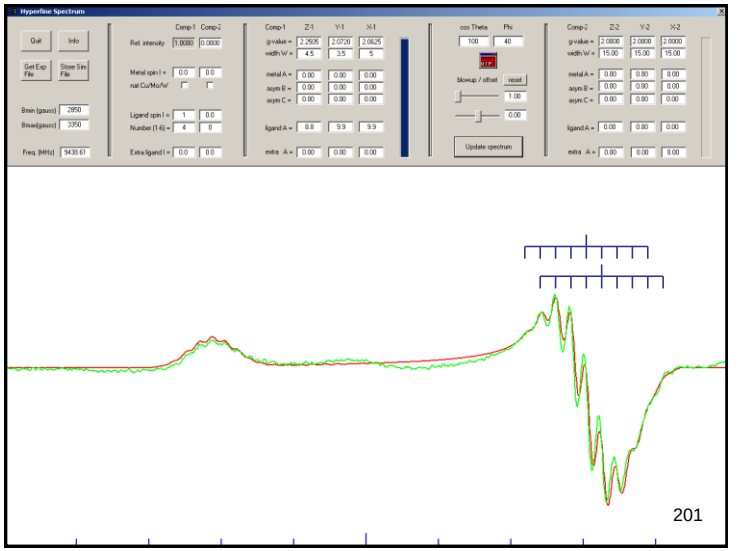


Simulations



MCRox1 g_{xyz} = 2.2305, 2.1665, and 2.1530
W_{xyz} = 3.5, 4.0, and 4.0
A^{N}_{xyz} = 8.0, 9.7, and 9.7

MCRred1 g_{xyz} = 2.2495, 2.0720, and 2.0625
W_{xyz} = 4.5, 3.5, and 5.0
A^{N}_{xyz} = 8.8, 9.9, and 9.9



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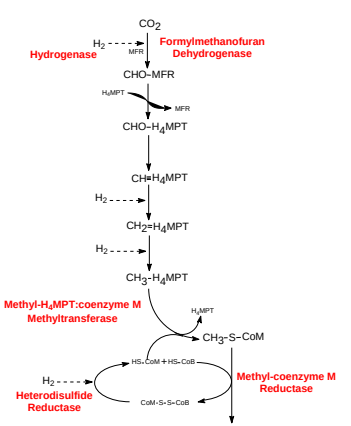
9) EPR on Whole Cells/Cell Extract

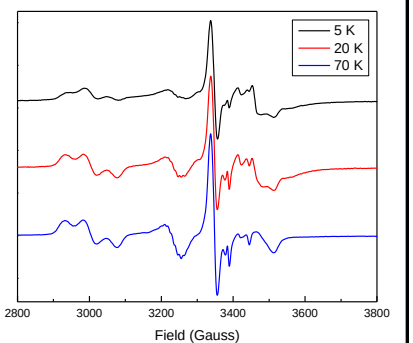
- CO_2-reducing pathway of methanogenesis, which uses H_2 and CO_2 as substrates.
- The reduction of CO_2 to CH_4 proceeds via coenzyme-bound C1-intermediates, methanofuran (MFR), tetrahydromethanopterin (H_4MPT), and coenzyme M (HS-CoM).



202

EPR on Whole Cells

- Overview of all paramagnetic species
- Behavior under different growth conditions
- Estimates of the amount of species present (simulations and integration)



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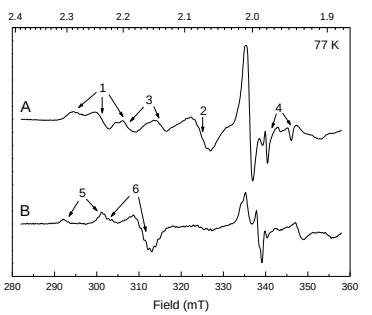
EPR on Whole Cells

A: 80% H_2/20% CO_2

B: 80% N_2/20% CO_2



1) MCR (red2 form)
2) MCR (red1 form)
3) Hydrogenase (Ni-C form)
4) Heterodisulfide reductase
5) Hydrogenase (Ni-A form)
6) MCR (ox1 form)

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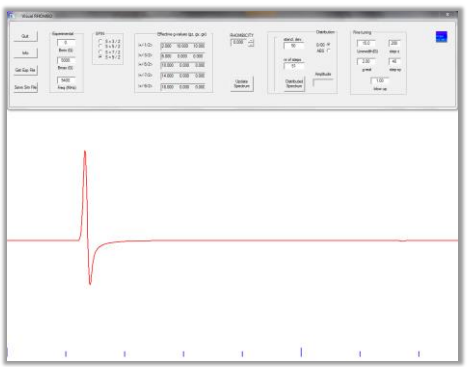
10) Visual Rhombo



- Estimates of the effective *g*-values

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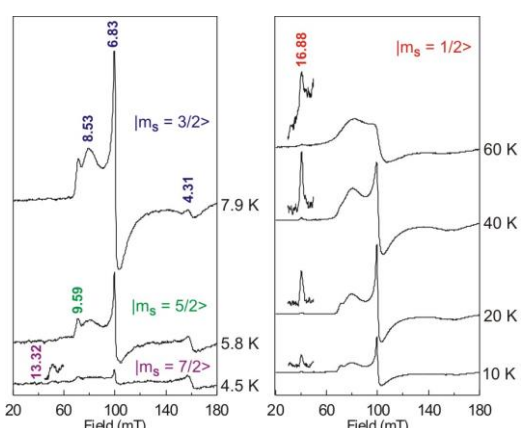
Simulation

Clostridium pasteurianum
$[2Fe\text{-}2S]^{2+}$

S = 9/2

(D < 0)



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