MOSBRI
Molecular-Scale Biophysics
Research Infrastructure

MOSBRI EPR School

Introduction to EPR Spectroscopy and Applications in Biology

Bruno GUIGLIARELLI

Unité de Bioénergétique et Ingénierie des Protéines – UMR 7281

CNRS et Aix-Marseille Université

cnrs
Infranalytics
Aix*Marseille université

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 101004806

Summary

1- EPR Basic principles:

- Basic principles of Magnetism
- Resonance phenomenon and EPR
- EPR detectable systems
- Free Radicals : Hyperfine interactions

2- Examples of Applications:

- Study of oxidative stress – ROS and RNOS, Spin trapping
- Radical enzymes: PFOR, SAM radical proteins

3 – Improving EPR sensitivity:

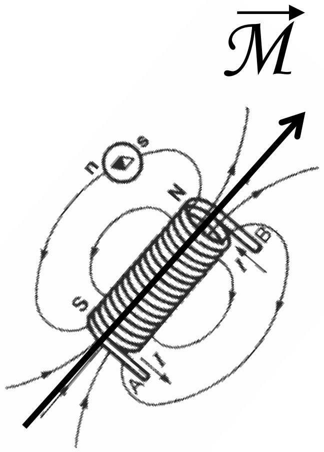
- Influence of T: Curie’s law,
- Field modulation
- Electron spin relaxation, Temperature dependence, relaxation broadening.

What is EPR (Electron Paramagnetic Resonance) ?

(also called ESR – Electron Spin Resonance)

A spectroscopy to study magnetic properties of matter

What is magnetism ?



Magnetism is related to motion of electric charges

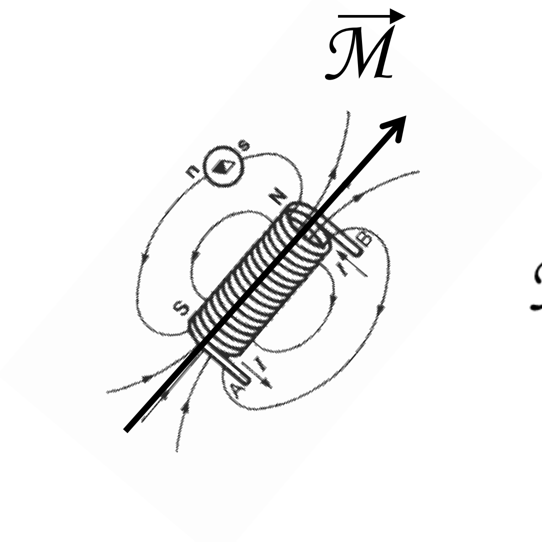
The magnetic dipolar moment

$$\vec{\mathcal{M}} = n\, I\, \vec{S} \quad (\mathrm{A{\cdot}m^2})$$

Compass

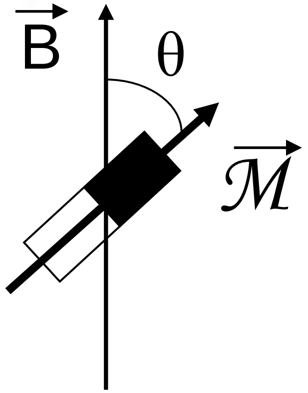
MOSBRI Molecular-Scale Biophysics Research Infrastructure

Basic principles of magnetism



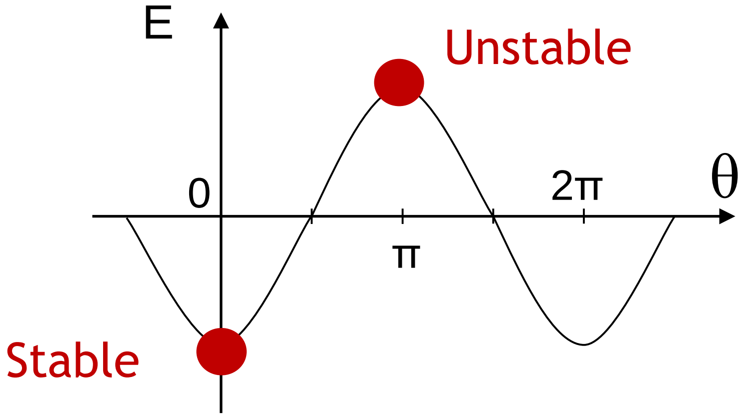
Magnetism is related to motion of electric charges

$$\vec{\mathcal{M}} = n\, I\, \vec{S} \quad (\mathrm{A \cdot m^2})$$

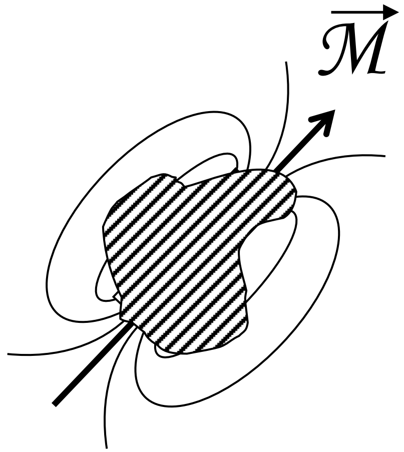


Energy of a magnet in a magnetic field

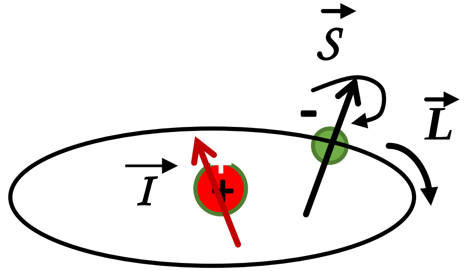
$$E = -\,\vec{\mathcal{M}} \cdot \vec{B} = -\,\mathcal{M} \cdot B \cdot \cos\theta$$



Basic principles of magnetism



In matter at atomic scale:
Moving charges are electrons and protons



- **Electron magnetism** :
  - ***L***, orbital momentum
  - ***S***, spin momentum

$\vec{\mu}_e = -\beta_e(\vec{L} + g_e\vec{S})$ $g_e = 2.0023$

$\vec{\mu}_e = -g\,\beta_e\,\vec{S}$ g = *Lande factor*

EPR

- **Nuclear magnetism**
  - ***I***, nuclear spin

$\vec{\mu}_N = g_N\,\beta_N\,\vec{I} = \gamma_N\,\hbar\,\vec{I}$

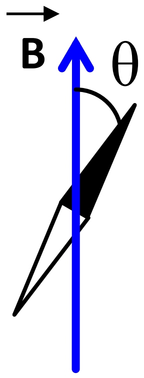
NMR

$\beta_e = e\,\hbar\,/\,2\,m_e = 9.274\cdot10^{-24}\ \mathrm{A\cdot m^2}$ **>>** $\beta_N = e\,\hbar\,/\,2\,m_P = 5.05\cdot10^{-27}\ \mathrm{A\cdot m^2}$

Bohr's magneton ~ 10^3 × Nuclear magneton

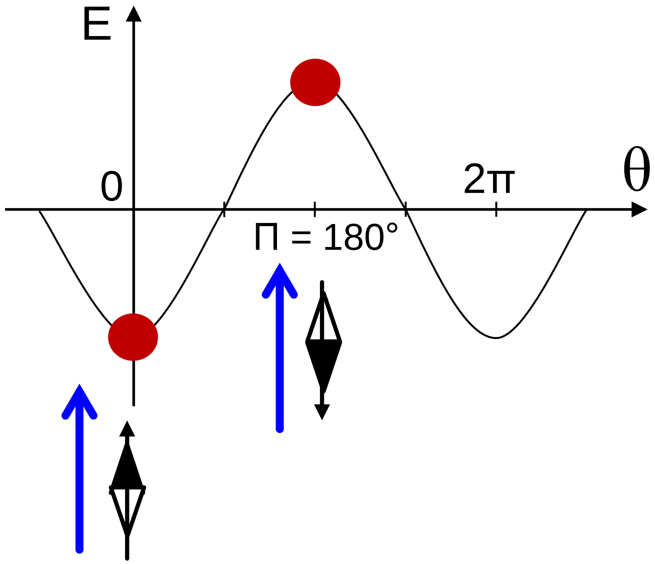
Basic principles of magnetism

Macroscopic scale



Energy of the compass in a magnetic field:

$$E = -\vec{M} \cdot \vec{B} = -M\,B\cos\theta$$

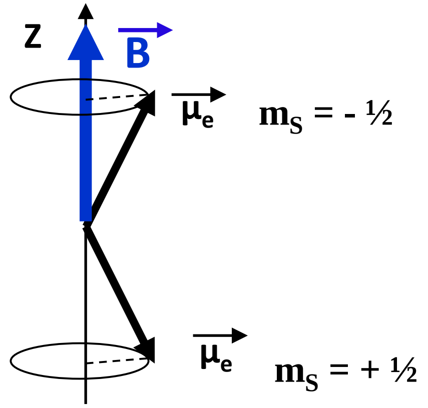


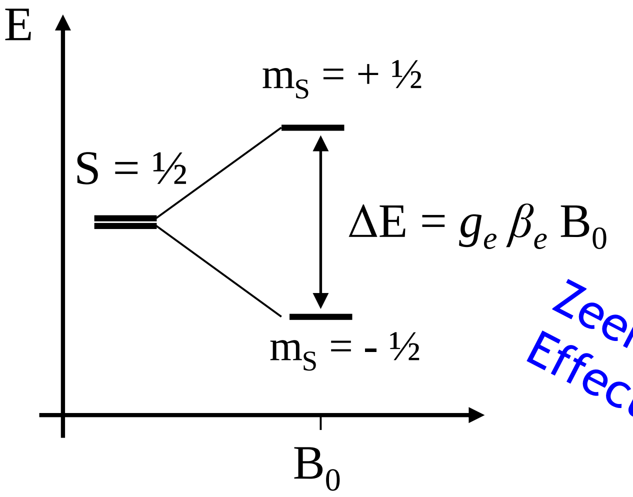
Microscopic scale: Quantum Physics

Electron spin : **S = ½**

Only two possible orientations of *S* in a magnetic field *B*: $m_s = +1/2\ ,\ -1/2$

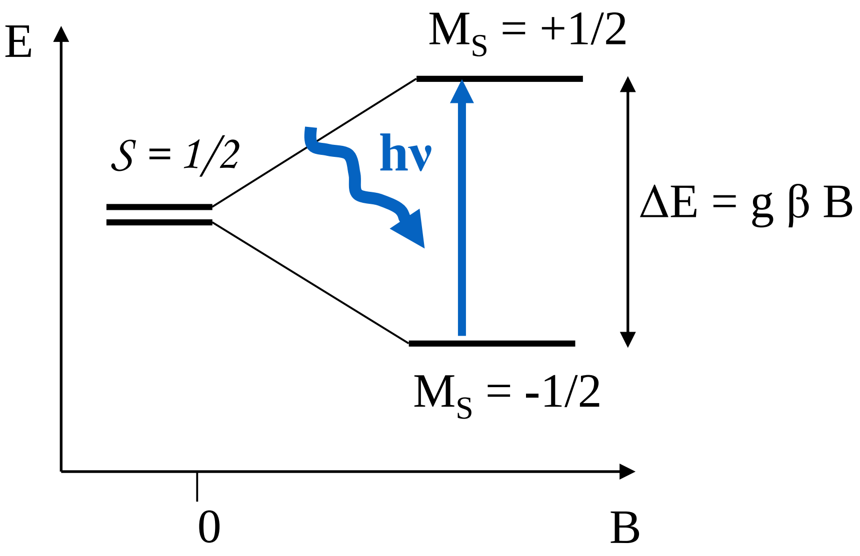
$$\vec{\mu}_e = -g\,\beta\,\vec{S}$$





Zeeman Effect

Resonance phenomenon and EPR

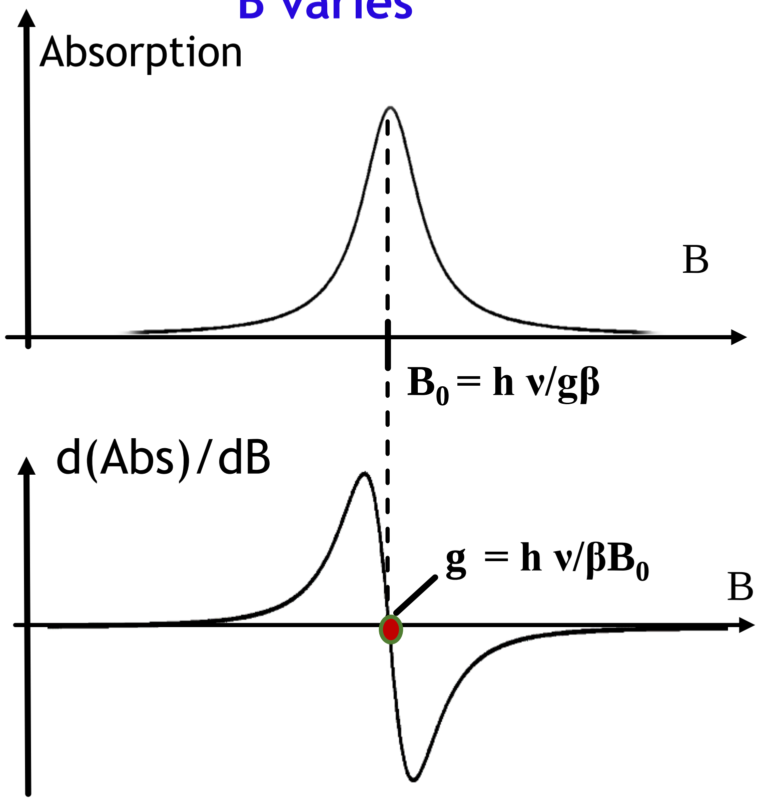


Resonance condition

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

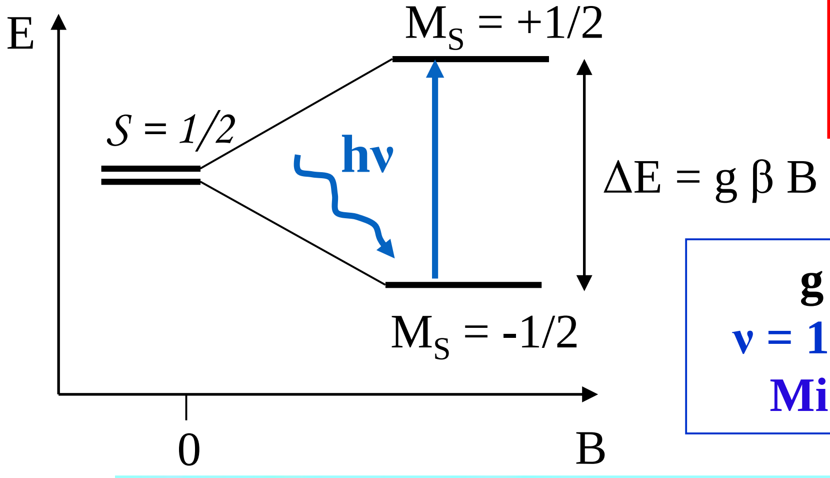
$g \sim 2.00$, $B_0 = 0.3$ T
$\nu = 10$ GHz, $\lambda = 3$ cm
Microwaves (X-band)

Frequency ν is fixed
B varies



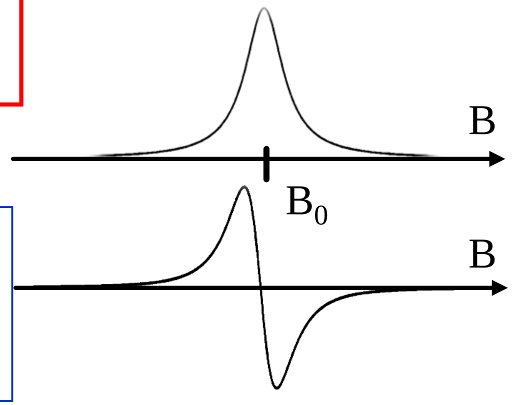
The experimental EPR spectrum is a derivative spectrum

Resonance phenomenon and EPR



Resonance condition:

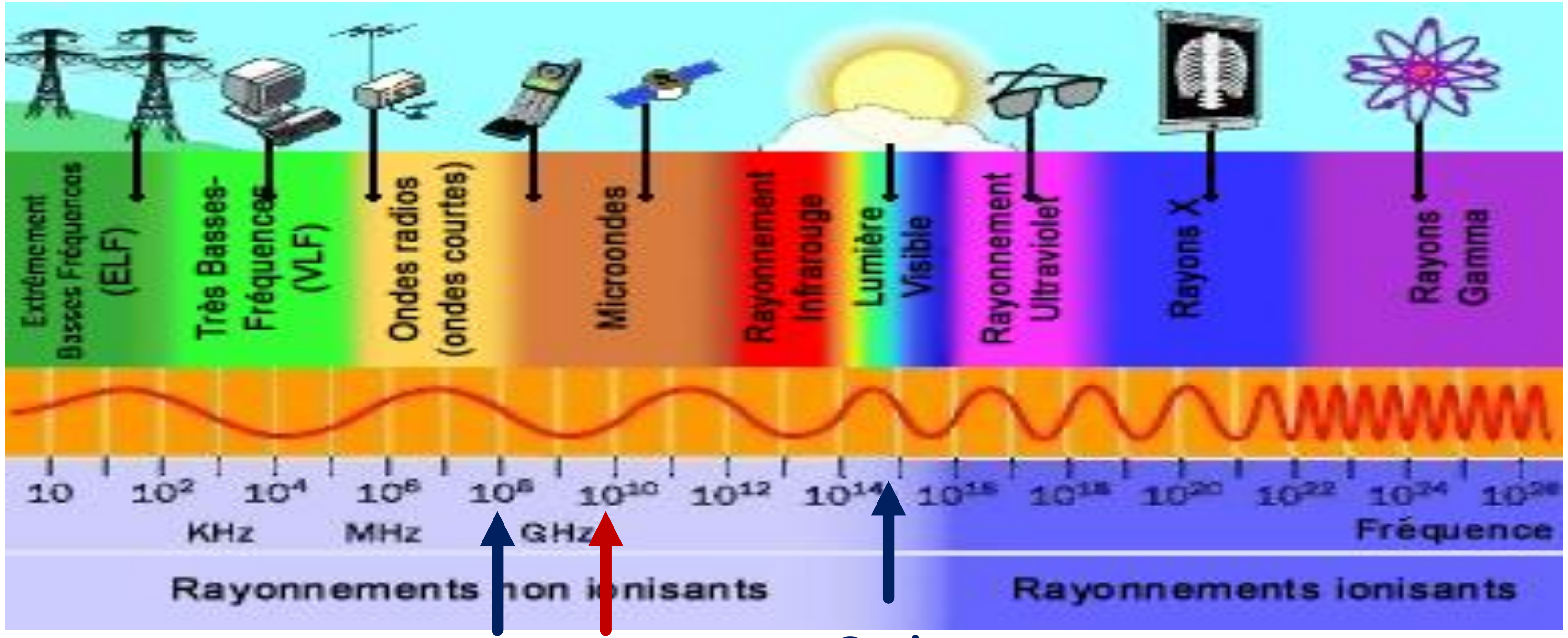
$h\nu = g\ \beta\ B_0$



$g = 2.00$, $B_0 = 0.3$ T

$\nu = 10$ GHz, $\lambda = c/\nu = 3$ cm

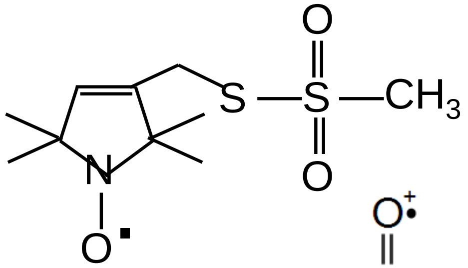
Microwaves (X-band)

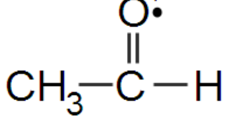


NMR **EPR** **Optics**

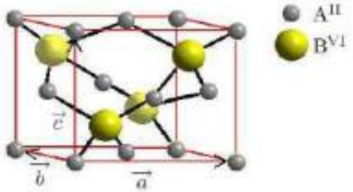
EPR detectable systems

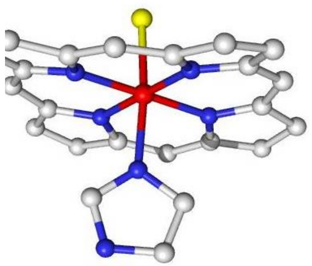
Presence of unpaired electrons $\mu_e \neq 0 \Rightarrow S \neq 0$



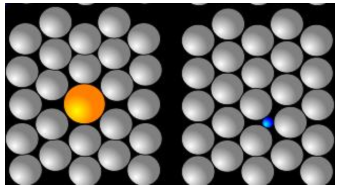


- **Odd electron number:**
  - Free radicals (organics, $OH^{\bullet}$, $NO^{\bullet}$, $NO_2^{\bullet}$, $HCO_3^{\bullet}$,…)
  - Transition metal ion compounds (Cu^{2+} , Fe^{3+} , Ni^{3+}, Mo^{5+}, V^{3+}, Ti^{3+},…) (open shell *d* orbitals)

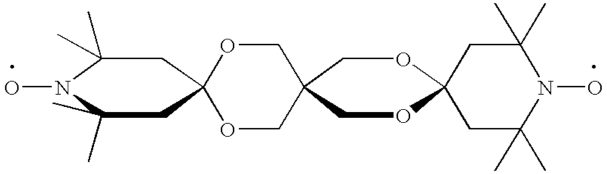




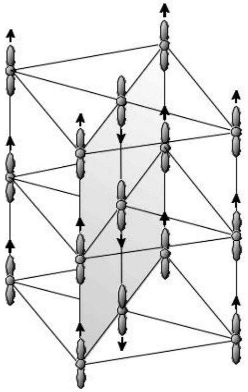
  - Impurities (doping) and defects in solids



- **Even electron number:**
  - Triplet states (excited or not), biradicals, O_2

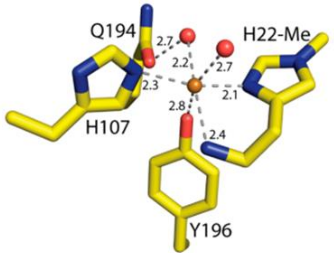


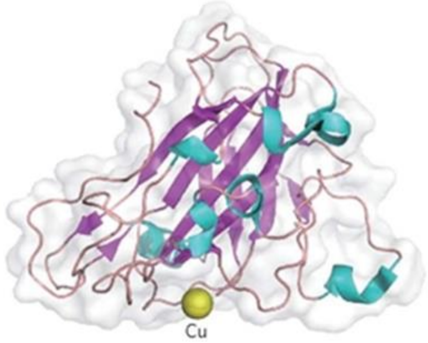
  - Conduction electrons, organic/inorganic molecular conductors, ferromagnets,….



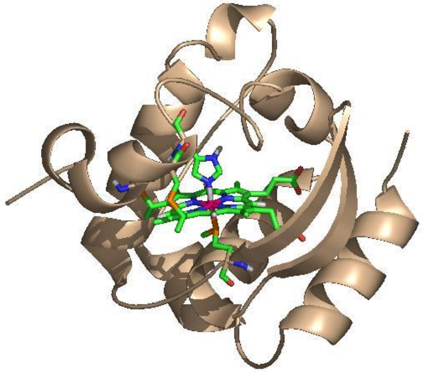
Paramagnetic centers in biology

Cu centers

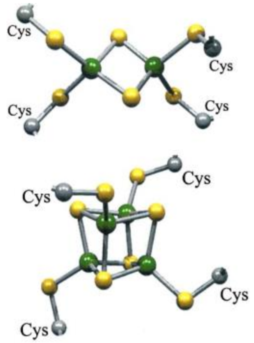


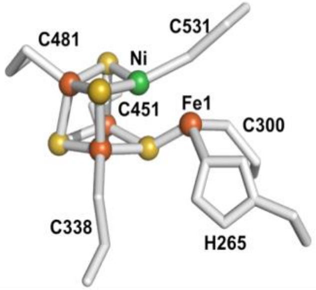


Hemes (cytochromes)

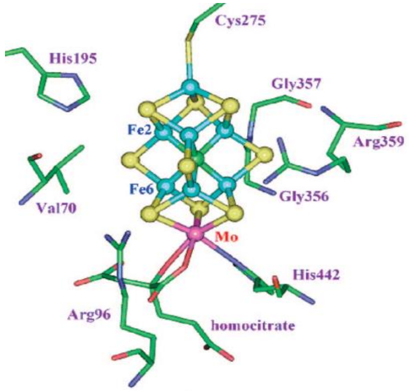


Fe-S clusters



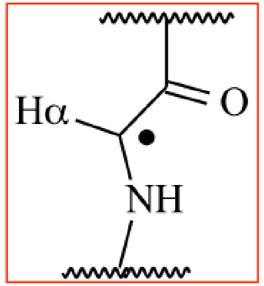


CODH : $NiFe_4S_4$

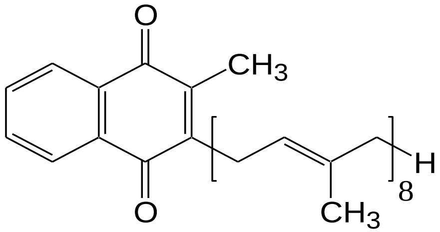


Nitrogenase : FeMo Cofactor

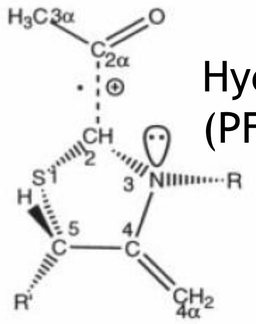
Radical intermediates



Glycyl radical

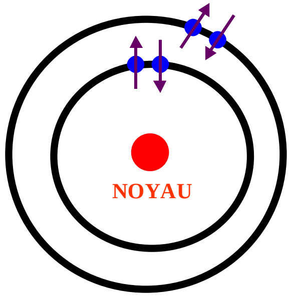


Semiquinones



Hydroxyethyl TPP (PFOR)

Organic free radicals



In most of molecules, all electrons are paired, with antiparallel spins in orbitals (Pauli principle) $\Rightarrow$ **S = 0** **EPR silent**

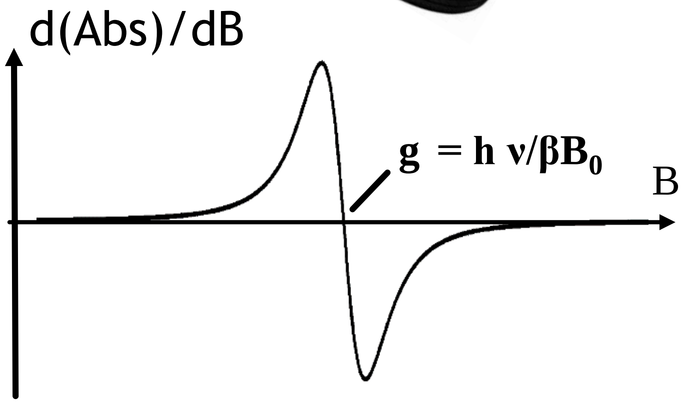
- Homolytic breaking of chemical bonds leads to the generation of free radicals

$$R{-}R' \longrightarrow R^{\bullet}\downarrow + R'^{\bullet}\uparrow$$

S = 0 **S = ½** **S = ½**

This bond breaking can be due to :
- Chemical reaction
- Redox reaction : Ox + e^- = Red
- Irradiation (X-ray, γ-ray, UV-vis)
- Thermal effect
- Mechanical strain (aging)

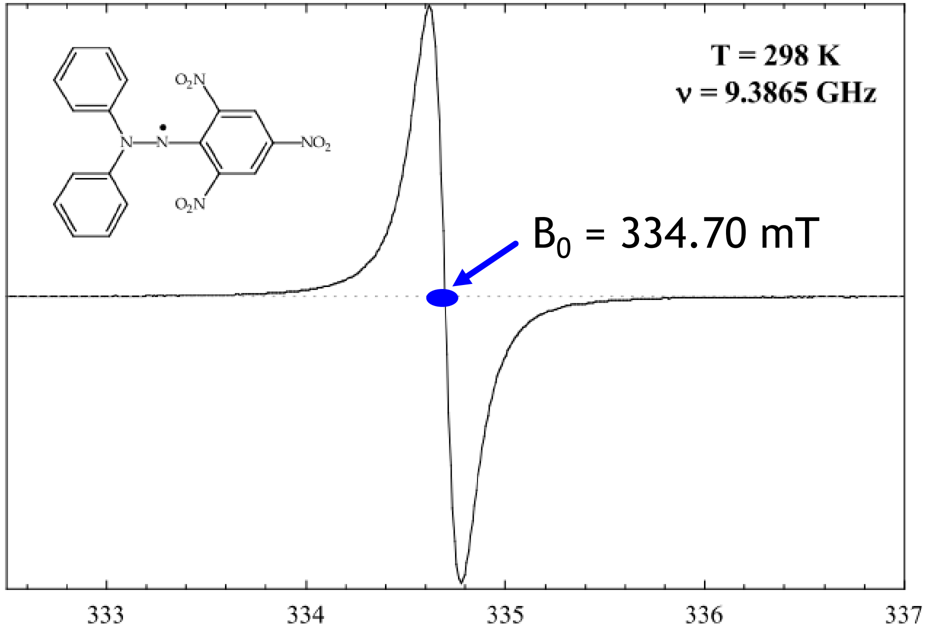
Free radicals with S = ½ are active in EPR :
- Detectable at room T, in solution
- Isotropic spectrum : $g \sim g_e = 2.00$
- But generally very reactive and transient species



MOSBRI Molecular-Scale Biophysics Research Infrastructure

Organic free radicals : some examples

Example of an EPR spectrum :
the case of *N,N*–diphenyl picrylhydrazyl (DPPH) radical



A stable radical used as a reference

$h = 6.626 \cdot 10^{-34}$ J.s
$\beta = 9.274 \cdot 10^{-24}$ J.T^{-1}
$B_0 = 334.70$ mT

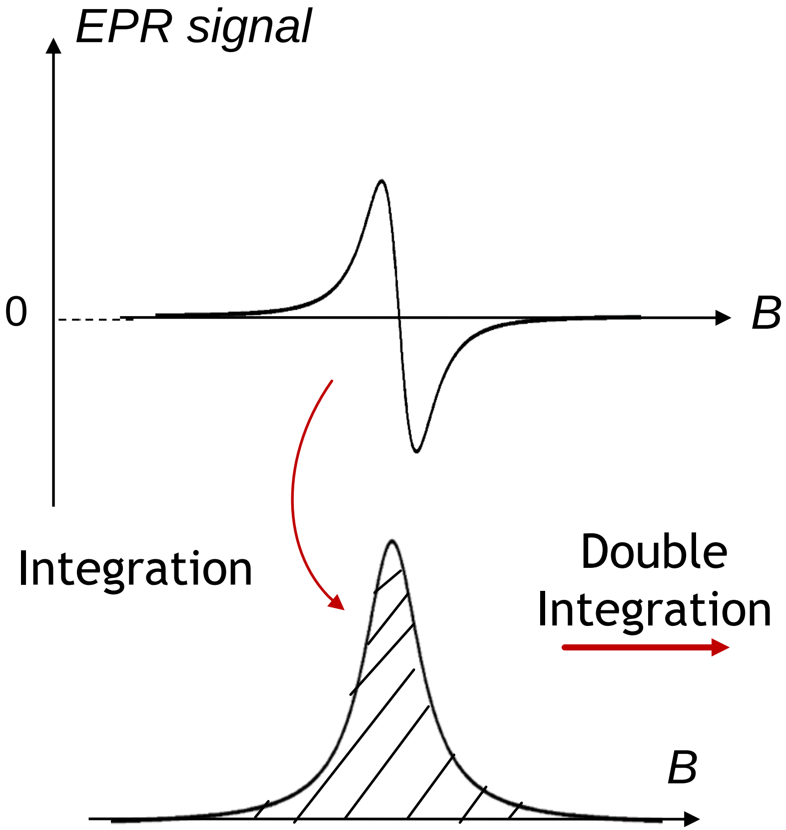
$g = h\nu / \beta B_0 = 2.0037$

For free electron: $g_e = 2.002\ 319\ 304\ (\pm 10^{-13})$

For radical centered on C, N, O:
$g - g_e \sim 10^{-4} - 10^{-3}$

Not easy to identify nature of radicals from g-value measurements alone

Organic free radicals – Spin intensity measurements



The area under absorption peak is proportional to I_{EPR}

Sensitivity

Line width: 1 Gauss (0.1 mT)

1 µM in 0.15 mL : 0.15 nmol

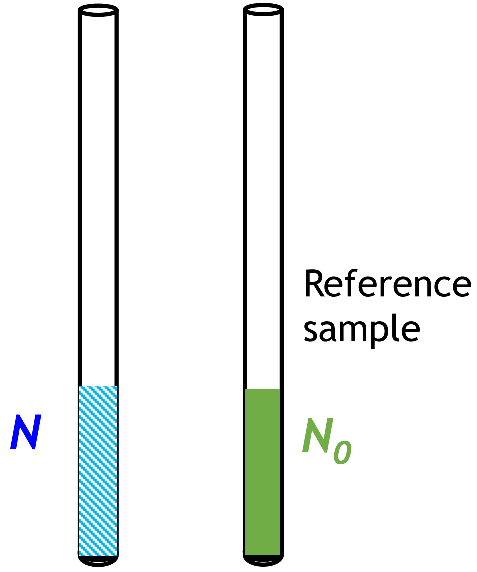
$N \sim 10^{14}$ spins

(with very thin lines: 10^{12} spins)

EPR signal intensity I_{EPR} :

$$I_{EPR} \propto N \sqrt{P_1}\, \Delta B_{mod}\, 1/T$$

- Titrations (pH, E, ligands)
- Kinetics studies
- Dosimetry (X-ray, γ-ray)



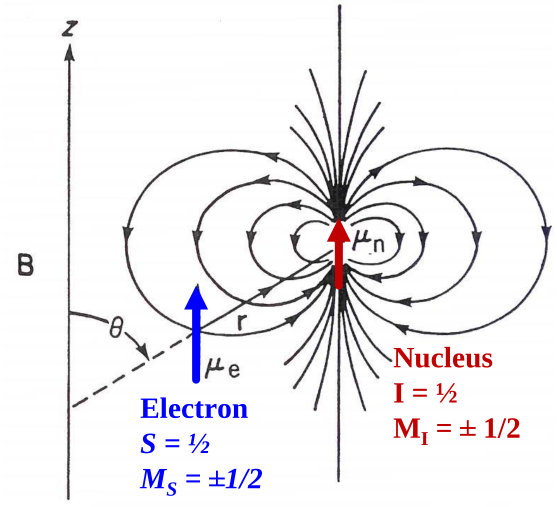
Quantitative measurements by comparison with a reference sample:

$$I_{EPR} / I_0 = N/N_0$$

Reference samples: same physical state:
- Strong pitch
- Stable nitroxide (TEMPO)
- Solution of $Cu(EDTA)^{2+}$

Organic free radicals : hyperfine interaction

Magnetic coupling between the electron spin *S* and the spin *I* of magnetic nuclei in the vicinity:
Dependence on distance and orientation (r, θ)
important structural information



Exemple with a proton ^1H in the vicinity
^1H nuclear spin *I* = ½
two spin states M_I = +1/2, -1/2

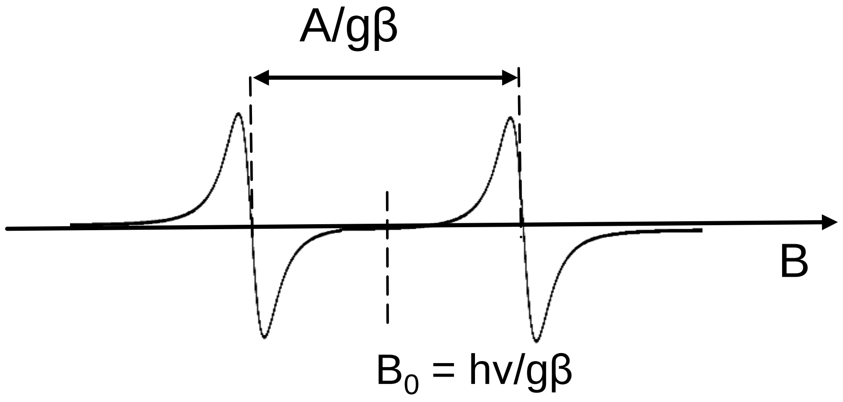
Splitting of the EPR line in **two** hyperfine lines

Hyperfine interaction hamiltonian

$$H = A\,\vec{S}\cdot\vec{I}$$

A is the hyperfine coupling constant

Splitting of the EPR line into
(2 I +1) = 2 hyperfine components

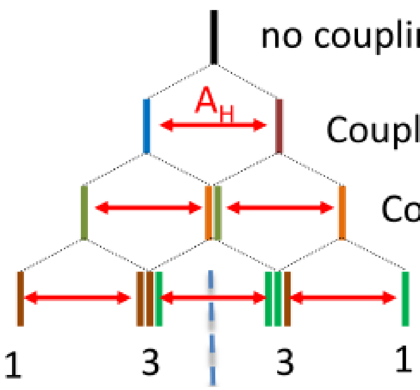


Nature and number of magnetic nuclei of the radical:
Structural identification

Organic free radicals : hyperfine interaction

As in NMR, multiple coupling with several nuclear spin is possible

Pascal's triangle



no coupling : one line

Coupling with 1 H : m_I = -1/2 ou m_I =+1/2 => 2 lines , separation =A_H

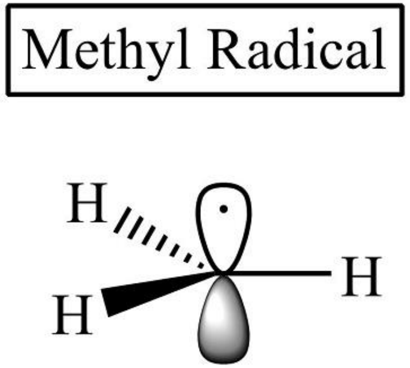
Coupling with a 2nd H (equivalent) : m_I = -1/2 ou m_I =+1/2 => 3 lines, separation A_H

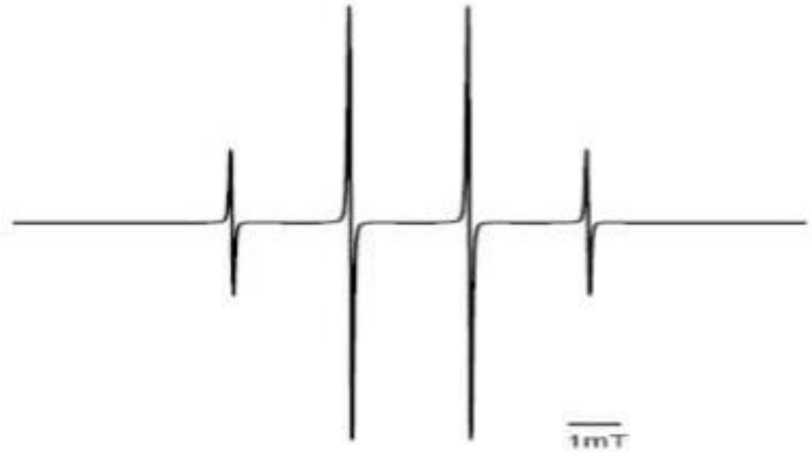
Coupling with 3rd H (equivalent) : m_I = -1/2 ou m_I =+1/2 => 4 lines, separation A_H

Relative intensity: 1 3 3 1

N equivalent ^{1}H give ***2^N*** hyperfine components leading to N+1 hyperfine lines

Example with $CH_3^•$



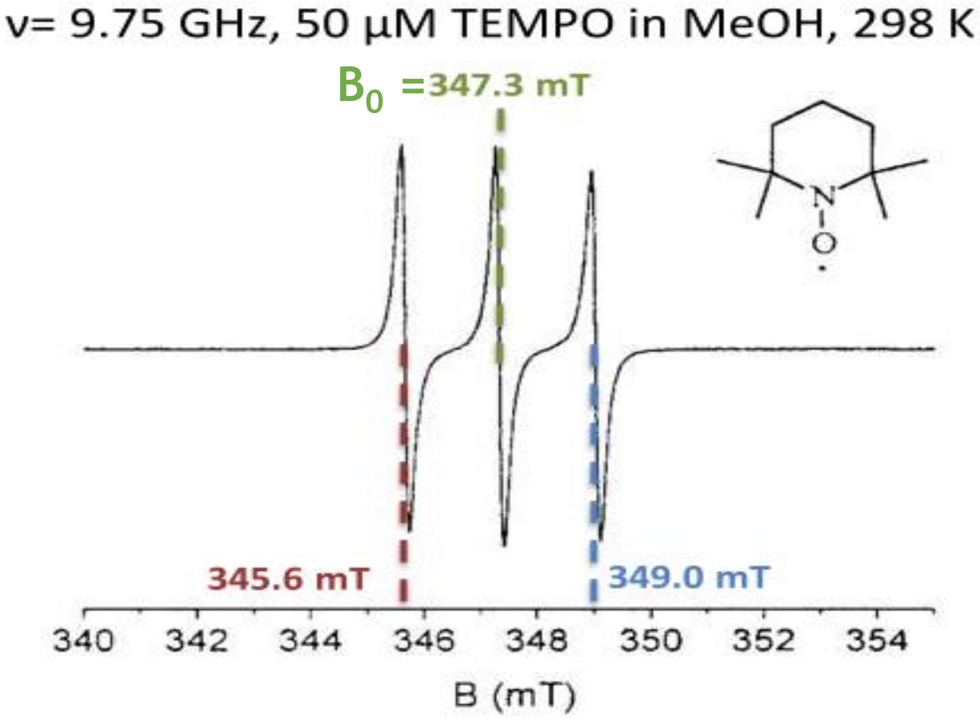


Organic free radicals : hyperfine interaction

Nitroxide radicals : R-NO•

Delocalisation of the electron spin (S =1/2) on the ^{14}N nucleus (I = 1):
I = 1, M_I = -1, 0, +1 (2I+1 values of M_I: M_I = - I, -I+1, -I+2,…., I)

2 I +1 = 3 hyperfine lines
Centered on $B_0 = h\nu/g\beta$ and separated by $\Delta B = A/g\beta$

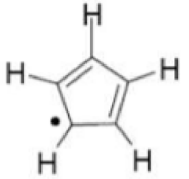


Hyperfine splitting:
$A/g\beta$ = 1.7 mT

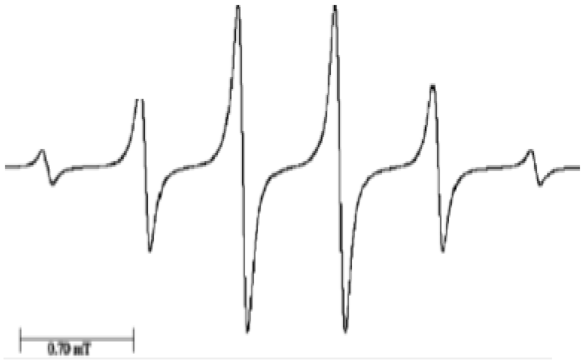
A can be expressed in MHz
A/h = 1.7 mT × $g\beta/h$
A/h = 47.6 MHz

(hyperfine couplings are much stronger than internuclei couplings in NMR)

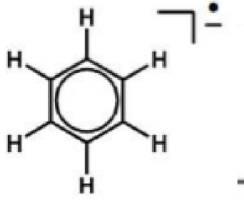
Organic free radicals : hyperfine interaction



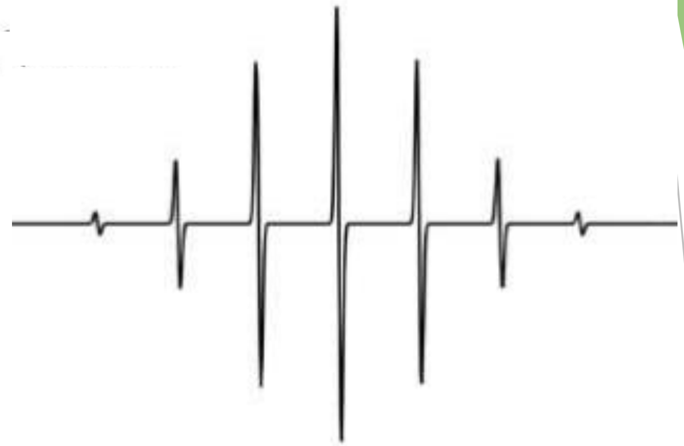
Cyclopentadienyl radical

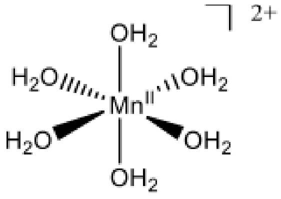


1
1 1
1 2 1
1 3 3 1
1 4 6 4 1
1 5 10 10 5 1
1 6 15 20 15 6 1
1 7 21 35 35 21 7 1



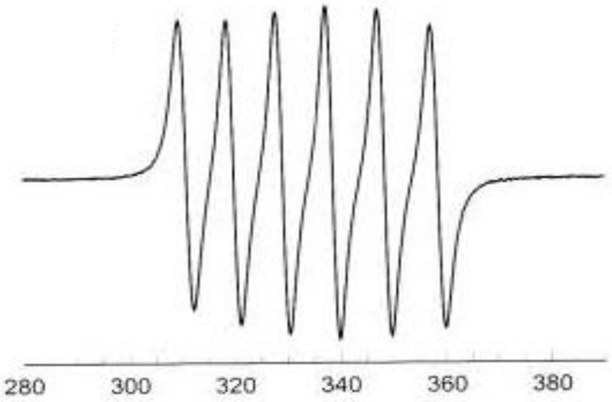
$C_6H_6^{\cdot -}$

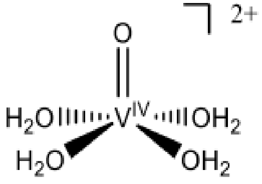




$Mn(OH_2)_6^{2+}$

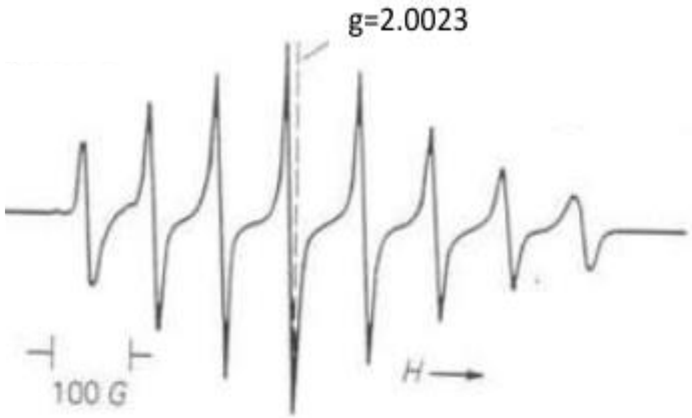
^{55}Mn, I = 5/2





$[V(O)(OH_2)_4]^{2+}$

^{51}V, I = 7/2

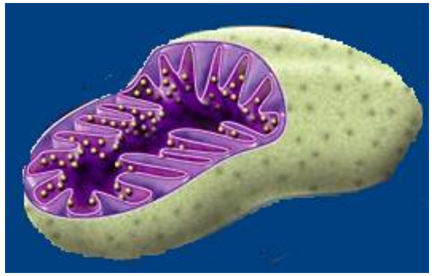


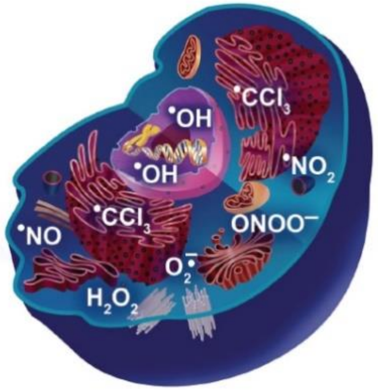
Application to the study of oxidative stress

Réactive oxygen and nitrogen species: ROS et RNOS

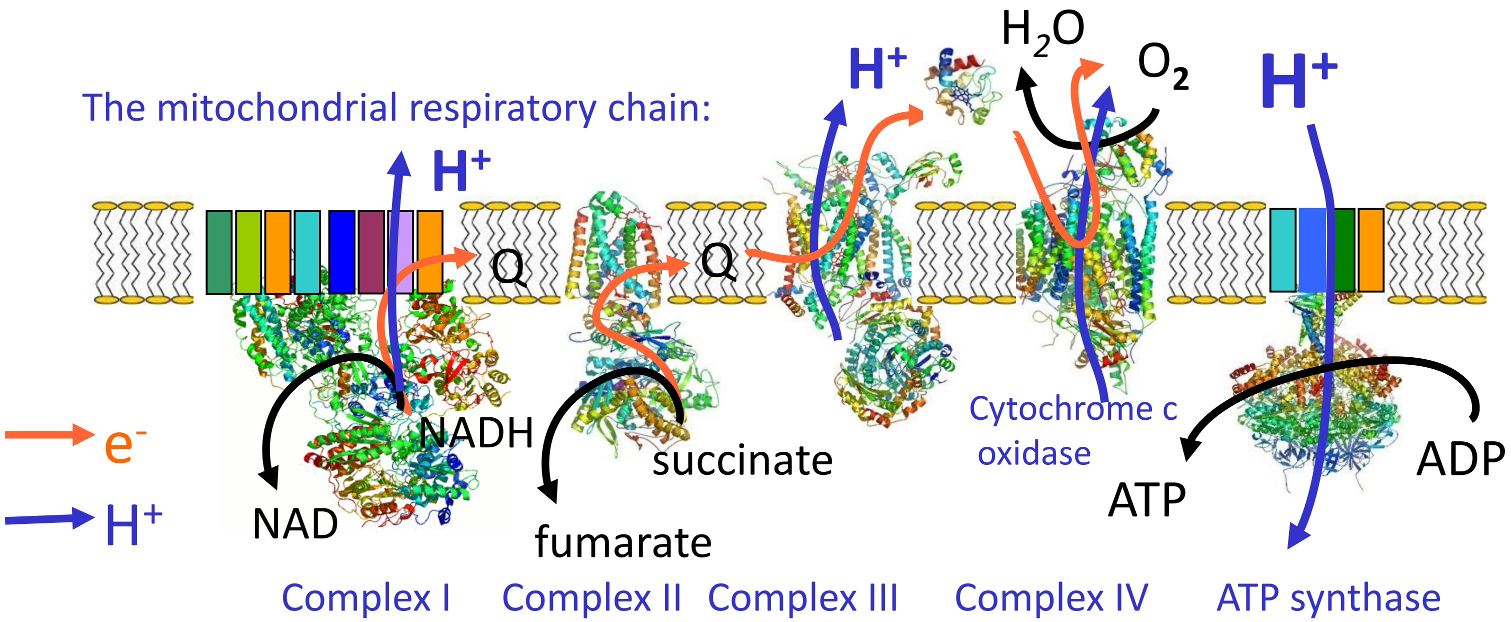
Mitochondrial respiration of O_2

$O_2 + 4\ e^- + 4\ H^+ = 2\ H_2O$





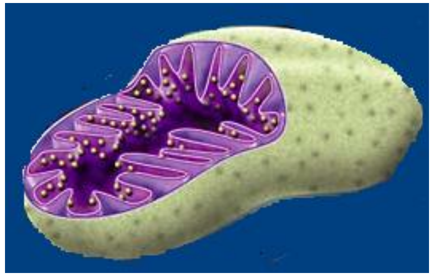
The mitochondrial respiratory chain:

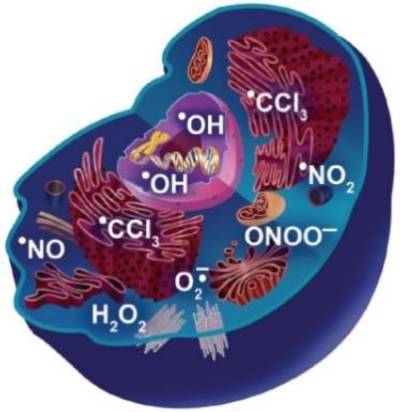


Application to the study of oxidative stress
Réactive oxygen and nitrogen species: ROS et RNOS

Mitochondrial respiration of O_2

$O_2 + 4\ e^- + 4\ H^+ = 2\ H_2O$





Problems due to electron sinks (NADH, Semiquinone : 5%)

$O_2 + e^- =$ $\mathbf{O_2^{\bullet -}}$ — Superoxide ion ⇒ oxidation of organic compouds

$O_2^{\bullet -} + e^- + 2\ H^+ =$ $\mathbf{H_2O_2}$ — Hydrogen peroxide ⇒ Fenton reaction

$H_2O_2 + Fe^{2+} = Fe^{3+} + OH^- + OH^\bullet$

$H_2O_2 + e^- + H^+ = H_2O +$ $\mathbf{OH^\bullet}$ — Hydroxyle ion extremely reactive ($k = 10^7 - 10^{10}\ M^{-1}\ s^{-1}$)

$O_2^{\bullet -} + NO^\bullet =$ $\mathbf{OONO^{\bullet -}}$ — Peroxynitrite ion

⇒ Many deleterious radical reactions:

Oxidation of catecholamines, thiols, hemoproteins, degradation of Fe-S centers,

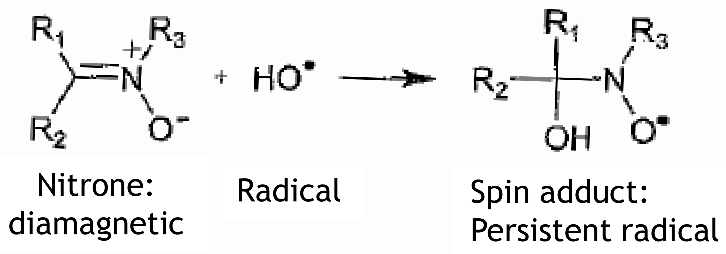
Lipid peroxidation (L-O-O•), clivage of protein chains, DNA, etc…

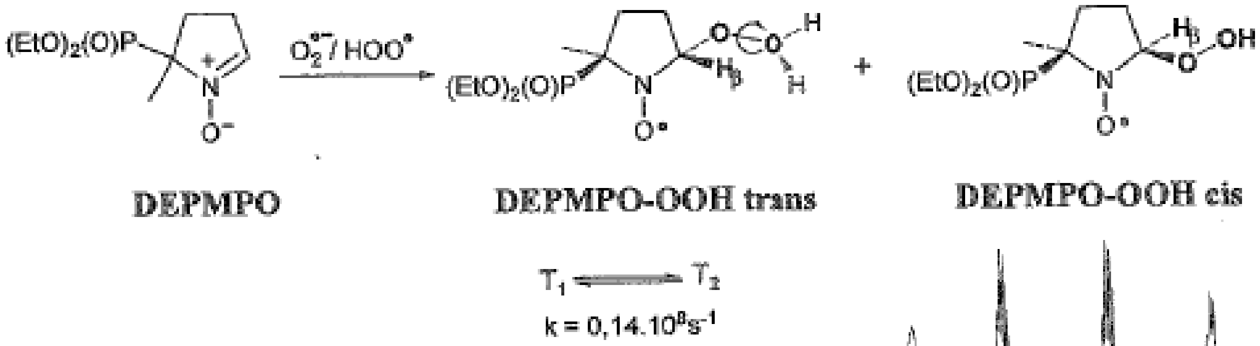
⇒ Dysfunction and cell death (apoptose)

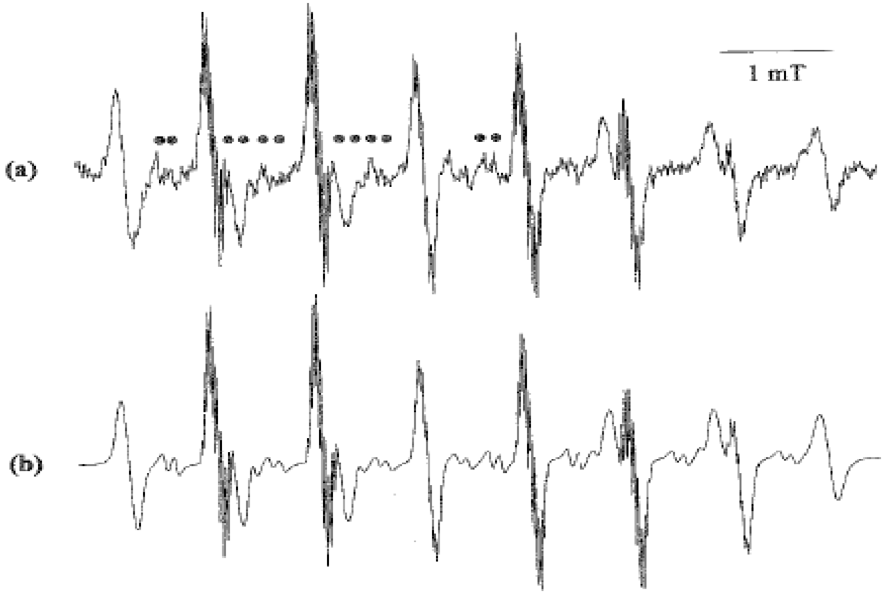
Applications to the study of oxidative stress

Transient radicals ⇒ Study by « spin-trapping » and formation of persistent radicals

Spin-traps : - Nitroso compouds R-N=O
- Nitrones



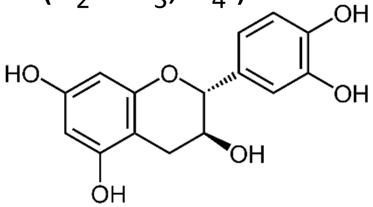


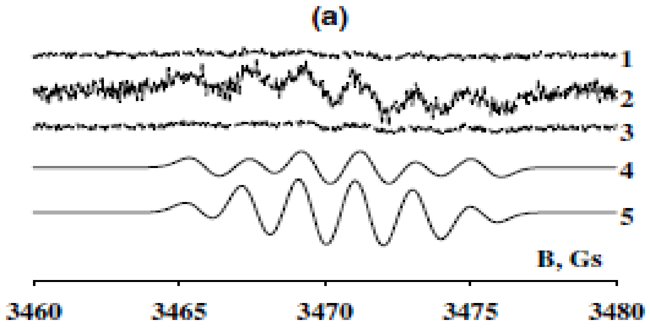


Oxidative stress: protection by polyphenols

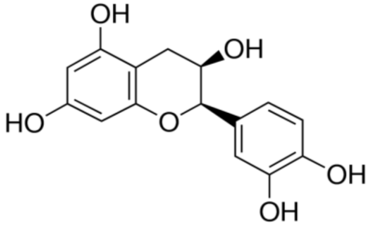
Antioxydant effect of wine polyphenols (flavonoïdes)
EPR/electrochemistry study

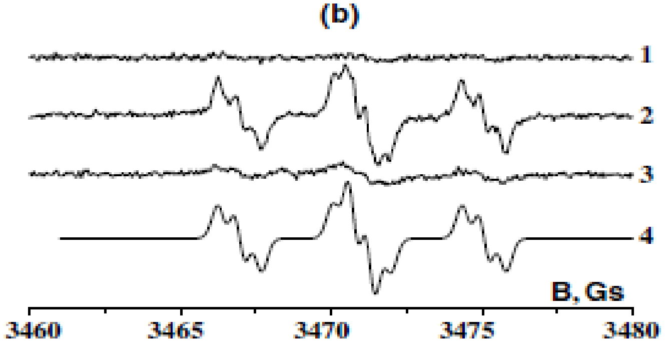
(+)-Catechine
($C_2 – C_3; C_4$)



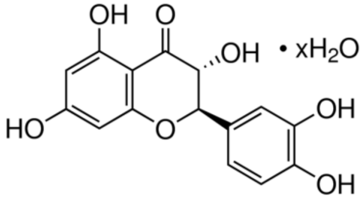


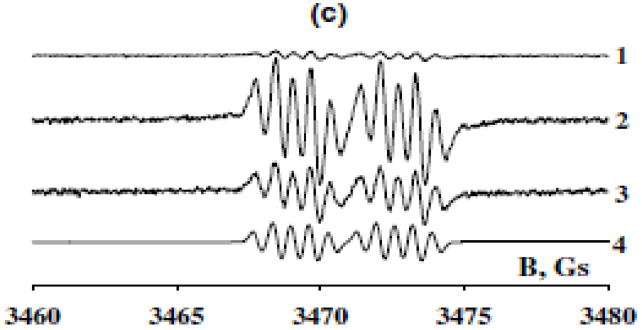
(-)-Epicatechine
($C_2 – C_3; C_4$)



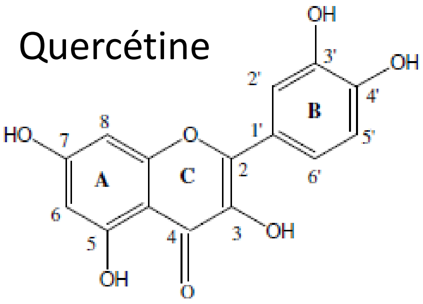


(+)-Taxifoline
($C_2 – C_3$)

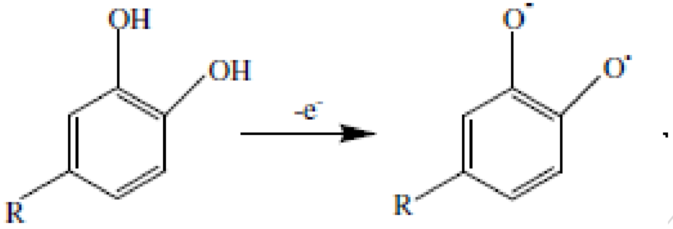




Quercétine



Stabilisation of the catechol radical



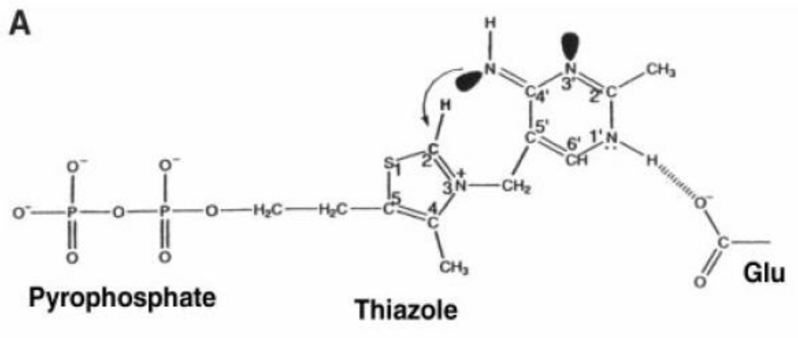
LeNest, 2004

Radical enzymes

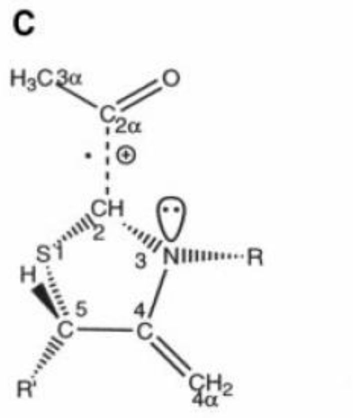
Radical intermediate in enzyme mechanism

PFOR : Pyruvate Ferredoxin Oxydoréductase
from anaerobic bacteria (*Desulfovobrio spp)*
CH_3–CO-COO^- + CoA = CO_2 + AcCoA + 2 e^-

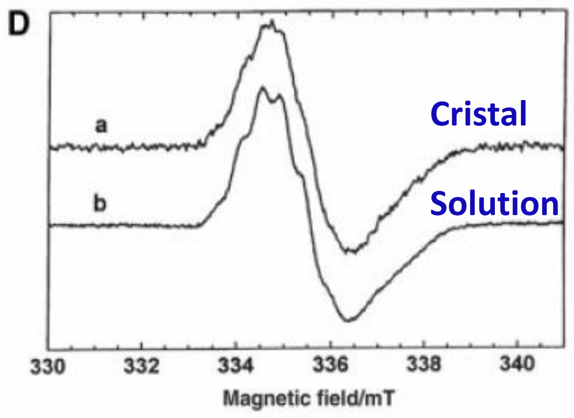
Active site: Thiamine PyroPhosphate TPP



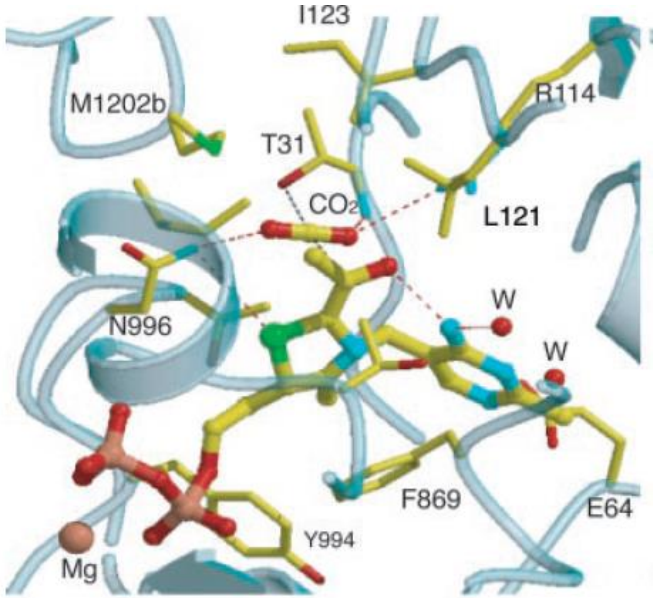
Identification of a radical intermediate and trapping in crystal state of PFOR



Hydroxyethyl-TPP radical
(Science, 2001)



$d(C_2–C_{2\alpha})$ = 1.95 Å (1 e^- bond)
Non-plannar TPP cycle

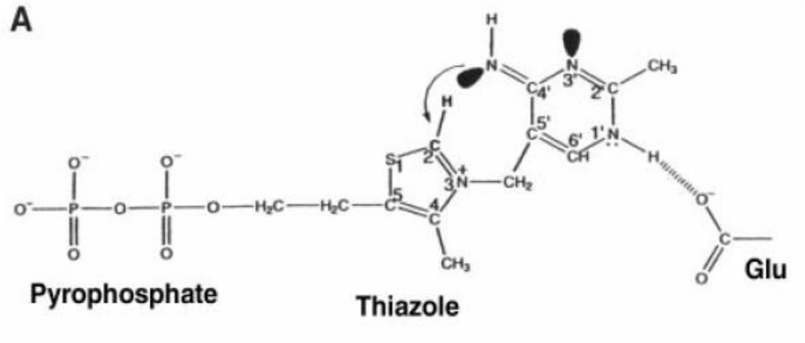


Radical enzymes

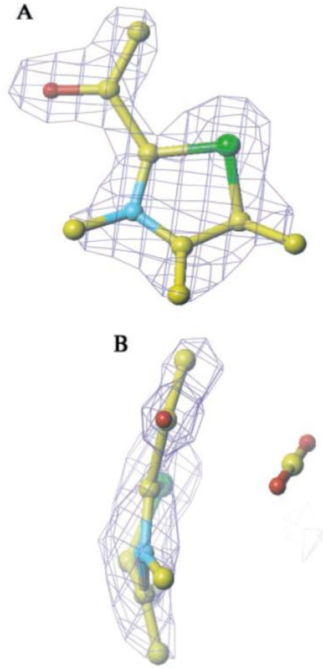
Radical intermediate in enzyme mechanism

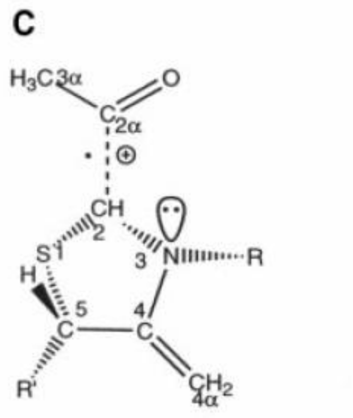
PFOR : Pyruvate Ferredoxin Oxydoréductase
from anaerobic bacteria (*Desulfovobrio spp)*
CH_3–CO-COO^- + CoA = CO_2 + AcCoA + 2 e^-

Active site: Thiamine PyroPhosphate TPP

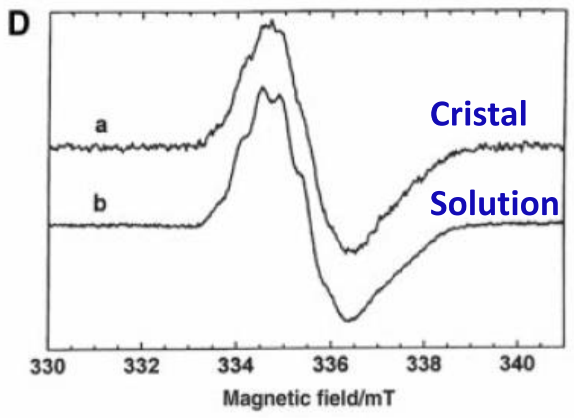


Identification of a radical intermediate and trapping in crystal state of PFOR





Hydroxyethyl-TPP radical
(Science, 2001)

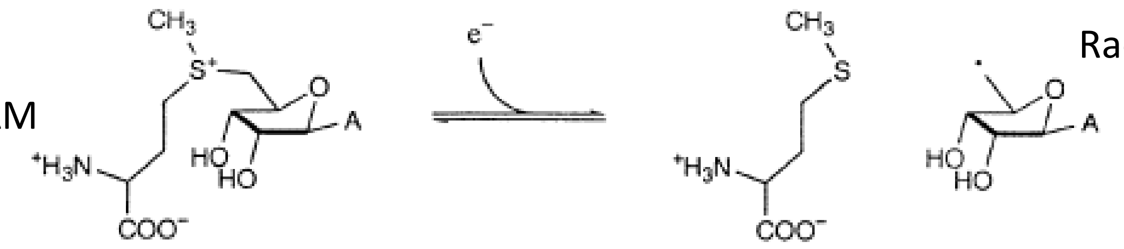


$d(C_2–C_{2\alpha})$ = 1.95 Å (1 e^- bond)
Non-plannar TPP cycle

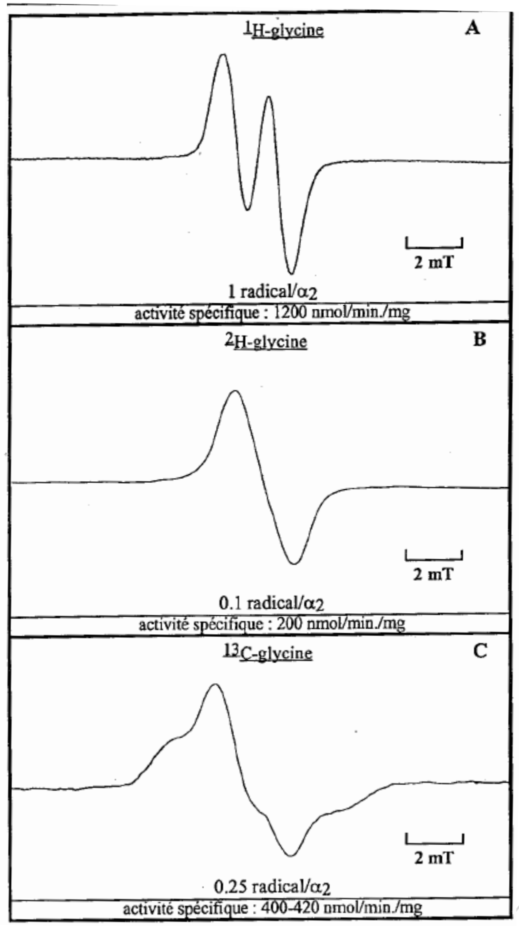
Radical enzymes

SAM-radical Enzymes family : use S-adenosylmethionine to generate a radical

SAM



Radical 5'-deoxyadenosyl

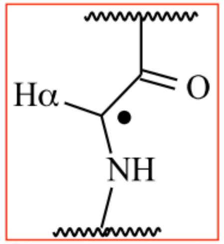


(Nicolet, *Nat. Catal*, 2020)

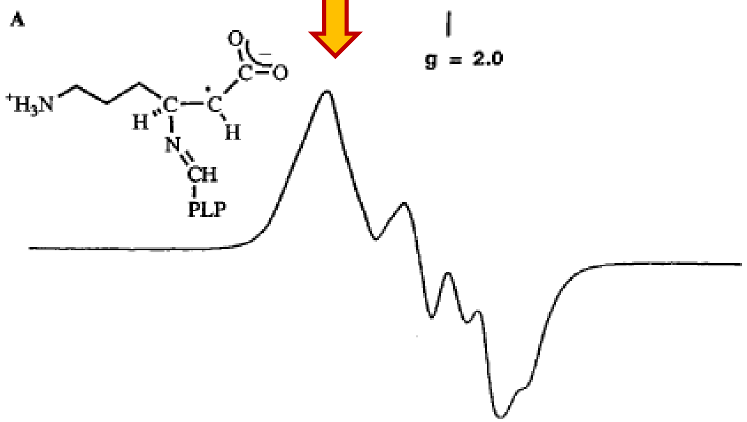
Generation of radicals on Glycine, Tyrosine, Cystéine

- Anaerobic Ribonucleotide reductase (RNR): Gly•
- Pyruvate formate-lyase (PFL)
- Biotine synthase

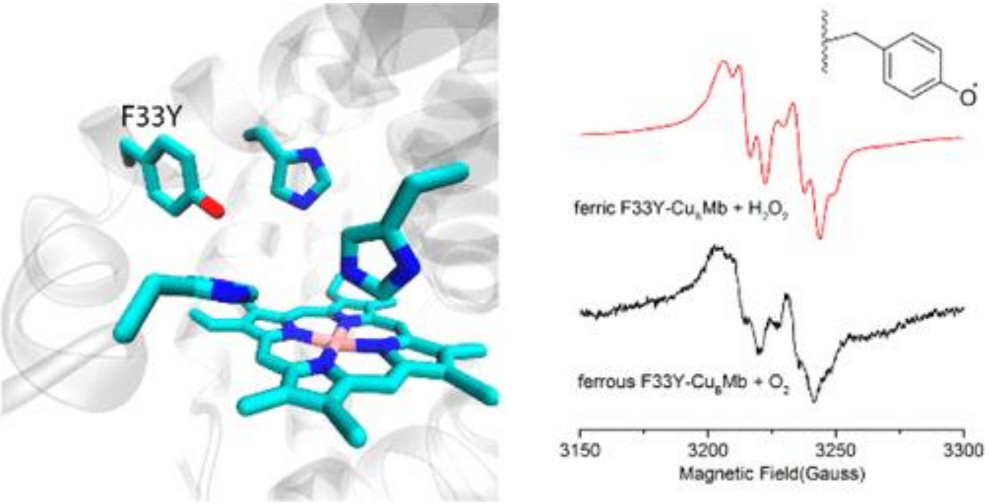
- Lysine 2, 3-amino mutase



Glycyl radical

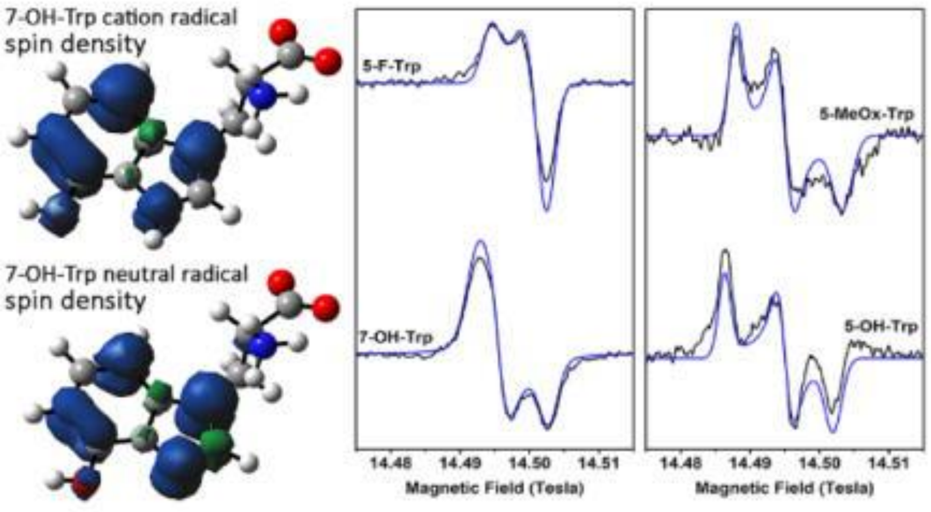


Radical enzymes

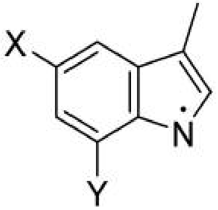


Tyrosyl radical:
Peroxidases, myoglobin mutants,…

Yu et al., JACS, 2014



Tryptophanyl radical

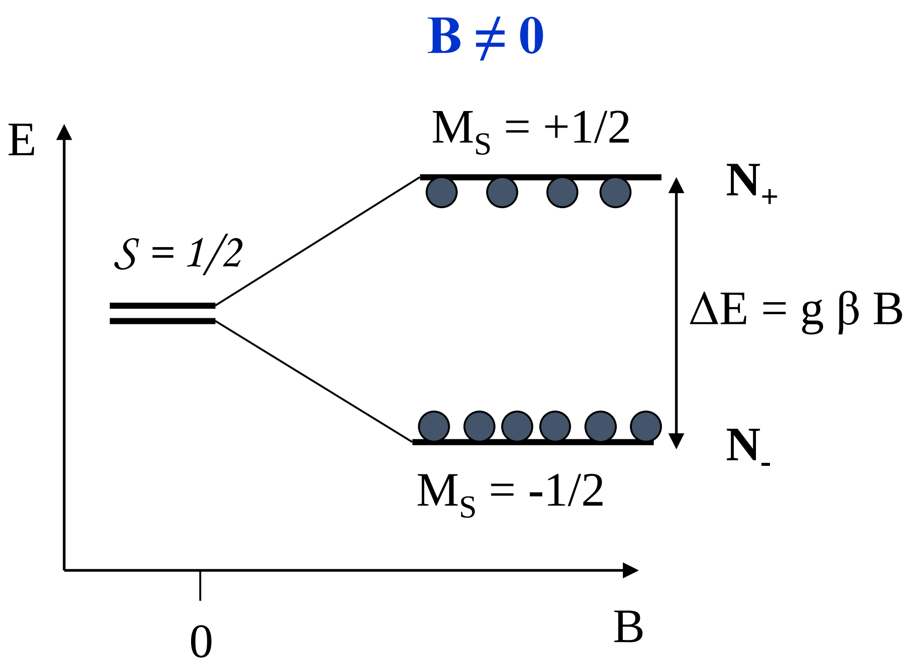


High frequency EPR: 406 GHz, 14.5 T

Davis et al., JPhysChem A 2018)

Improving EPR sensitivity - T dependence

Thermal equilibrium and spin state populations



Weak value of $\Delta E = g\,\beta\,B$

B= 0.3 T $\Delta E \sim 0.3\ cm^{-1}$

Thermal equilibrium (Boltzmann's law)

$$N_+ / N_- = \exp(-\Delta E / k_B T)$$

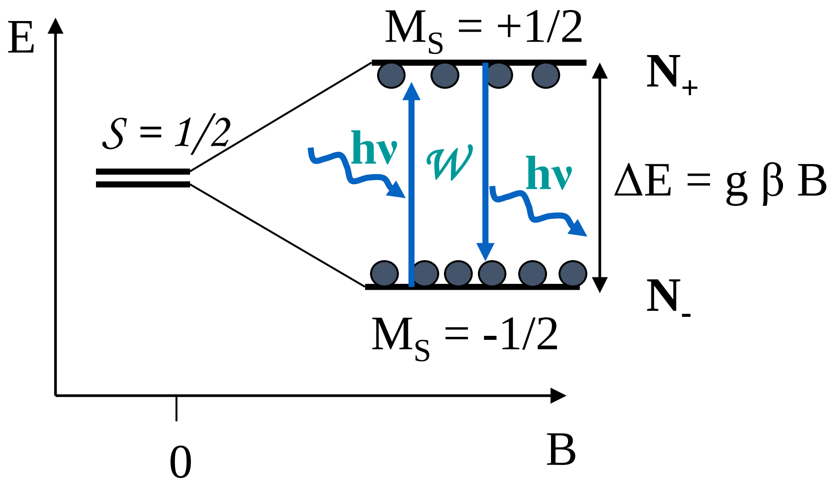
$$N_+ / N_- = \exp(-g\,\beta\,B / k_B T)$$

T=298 K, $N_+/N_- = 0.9986$

Very weak spin polarization

$$p = (N_- - N_+)/(N_- + N_+) = 7 \cdot 10^{-4}$$

Improving EPR sensitivity - T dependence



Microwave induced transitions

$B_1(t) = B_1 \cos(\omega t)$

Same transition probability for absorption and emission

$\mathcal{W} \propto B_1^2 \propto P_1$ (mW)

P_1 is the microwave power

EPR signal : net absorbed power

$P_{abs} = h\nu\,(\mathcal{W}N_- - \mathcal{W}N_+) = h\nu\,\mathcal{W}\,n$ with $n = N_- - N_+$

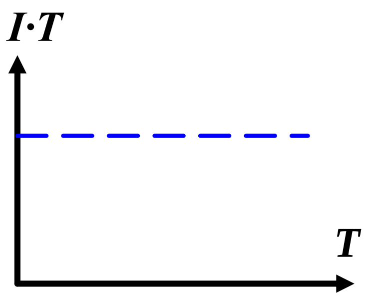
EPR signal intensity is directly related to the population difference *n*

Pierre Curie

Important consequence : Curie's law

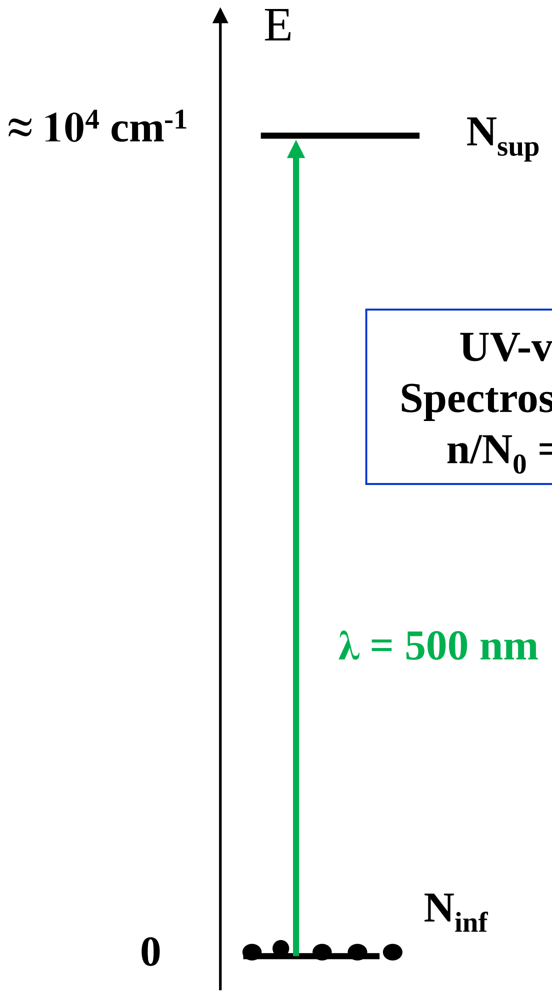
$I \propto n/N_0 = \text{th}(g\beta B_0/2k_BT) \approx g\beta B_0/2k_BT$

EPR signal intensity obeys the **Curie's law** $I \cdot T = Cte$



Energy absorption at resonance

$n = N_{inf} - N_{sup}$ **population difference**   **Signal** $\propto$ **n**



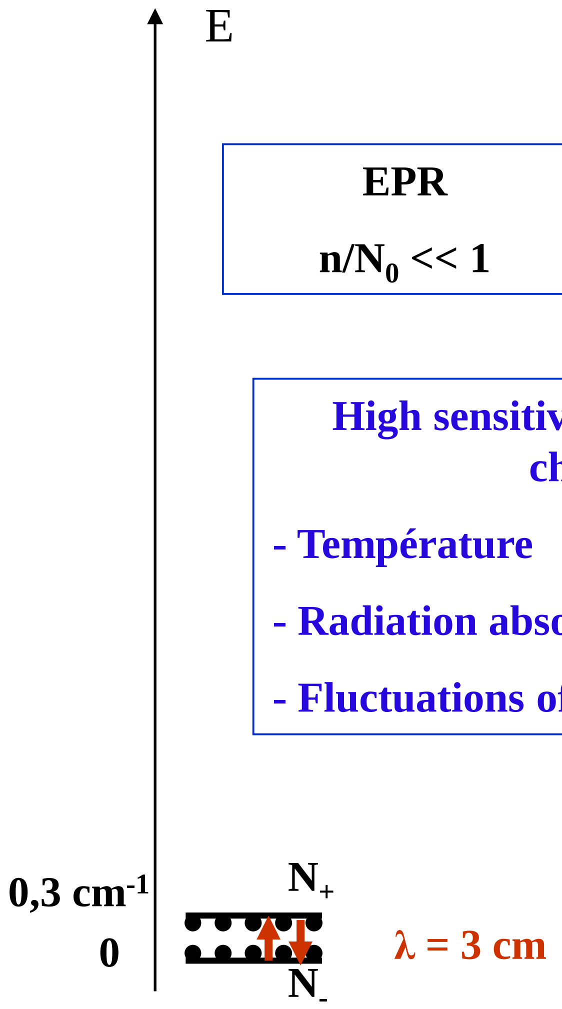
UV-vis Spectroscopy
$n/N_0 = 1$

EPR

$n/N_0 << 1$

High sensitivity to population changes

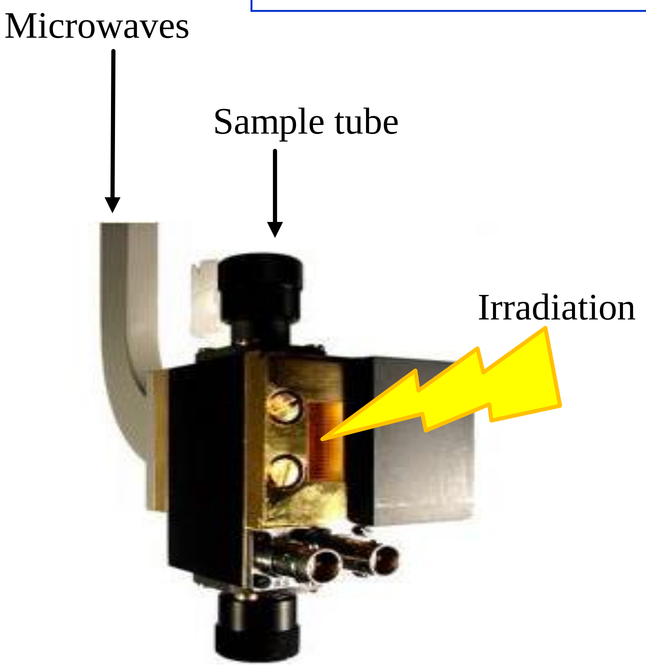
- **Température**
- **Radiation absorption**
- **Fluctuations of the environment**



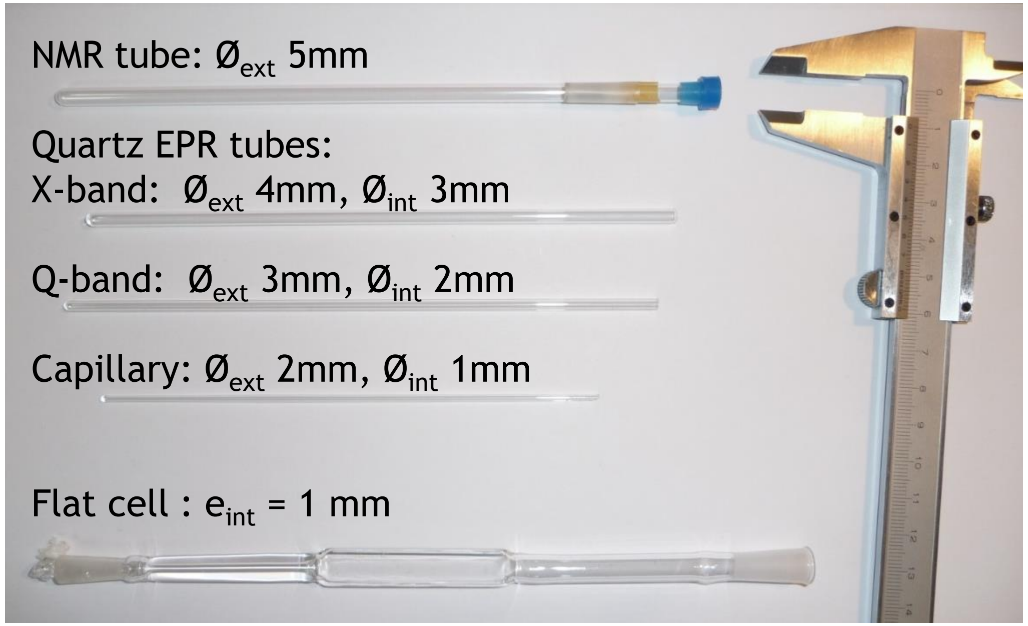
Improving EPR sensitivity : EPR cavity

EPR signal intensity: $\boldsymbol{I \propto N g\beta B / 2k_B T}$

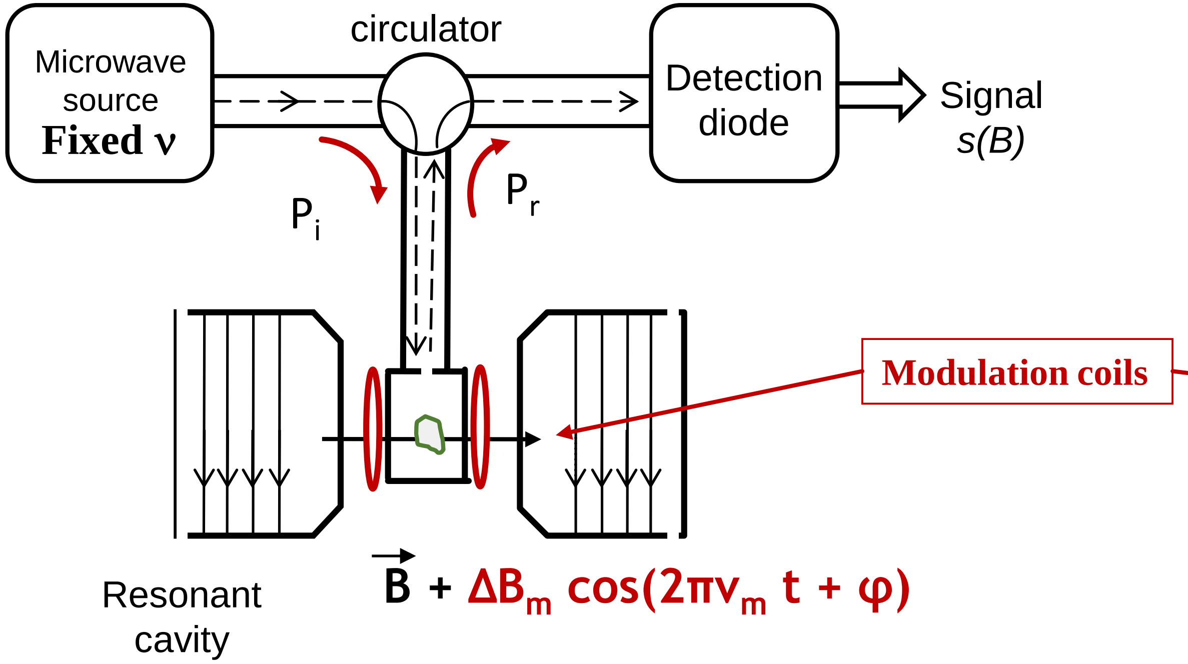
- Sample concentration (*N*, *number of spins*)
- Resonant cavity: Quality factor Q ~5-6000
- Low temperatures - cryogeny: liquid N_2 (77K), liquid He (4.2 K)
- High magnetic field / high frequency: Q-band 35GHz, W-band 95 GHz, …. 300 GHz

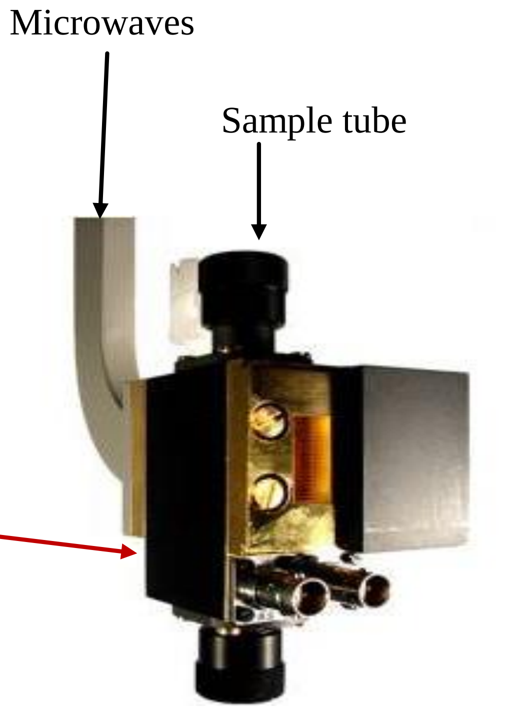


Rectangular standard cavity TE102

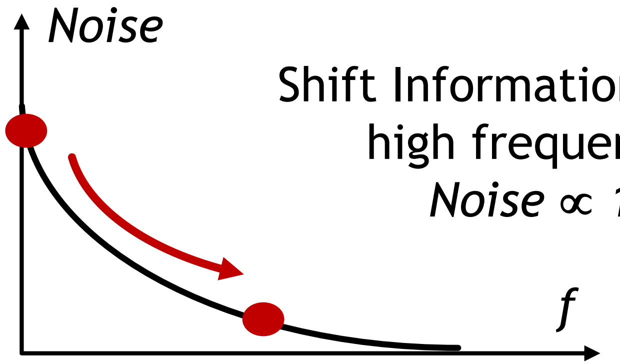


Improving EPR sensitivity: Modulation of the magnetic field





Rectangular cavity TE102

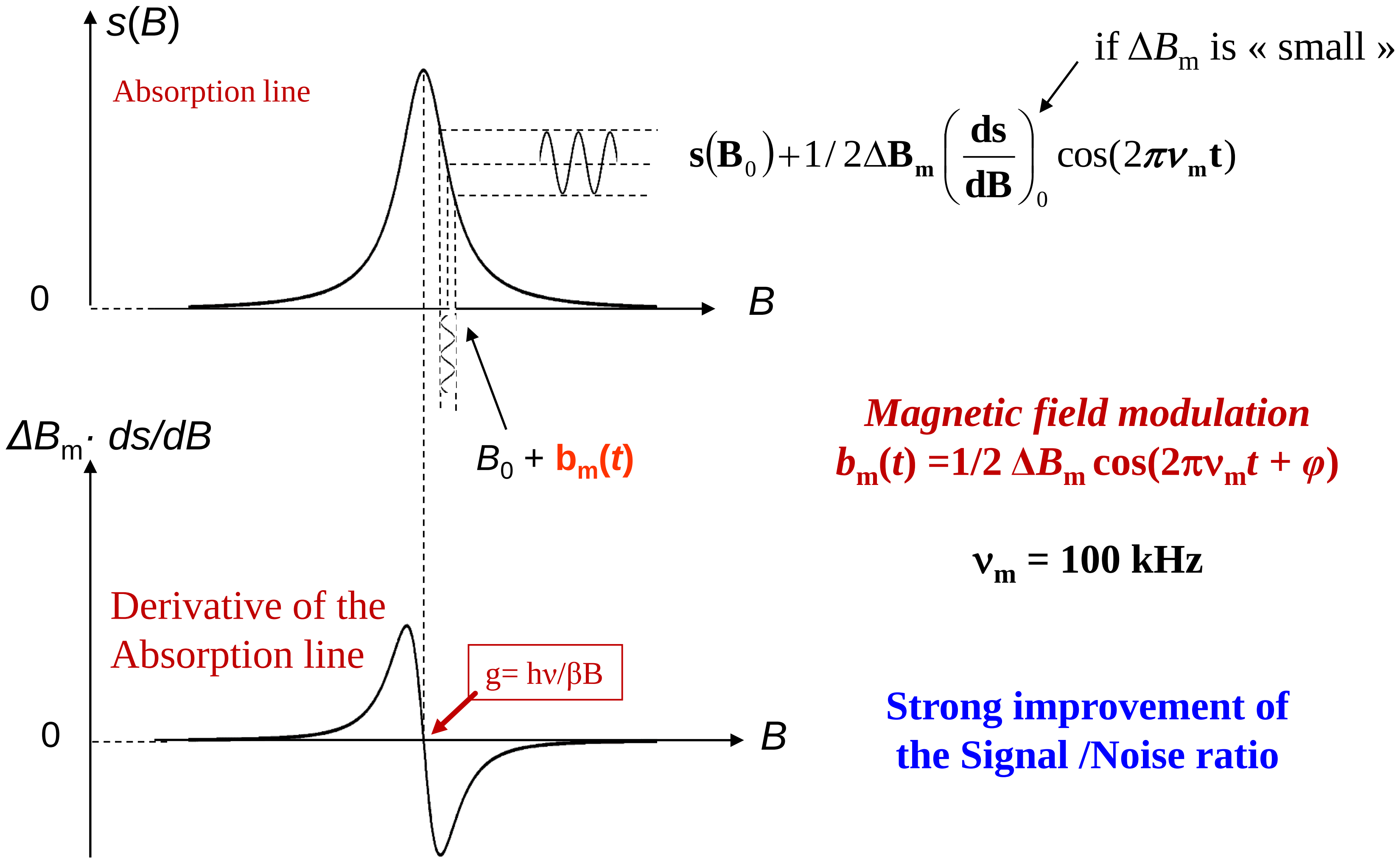


Shift Information towards high frequencies:

Noise $\propto 1/f$

Improving EPR sensitivity: Modulation of the magnetic field

Decrease the noise: Magnetic field amplitude modulation



Magnetic field modulation

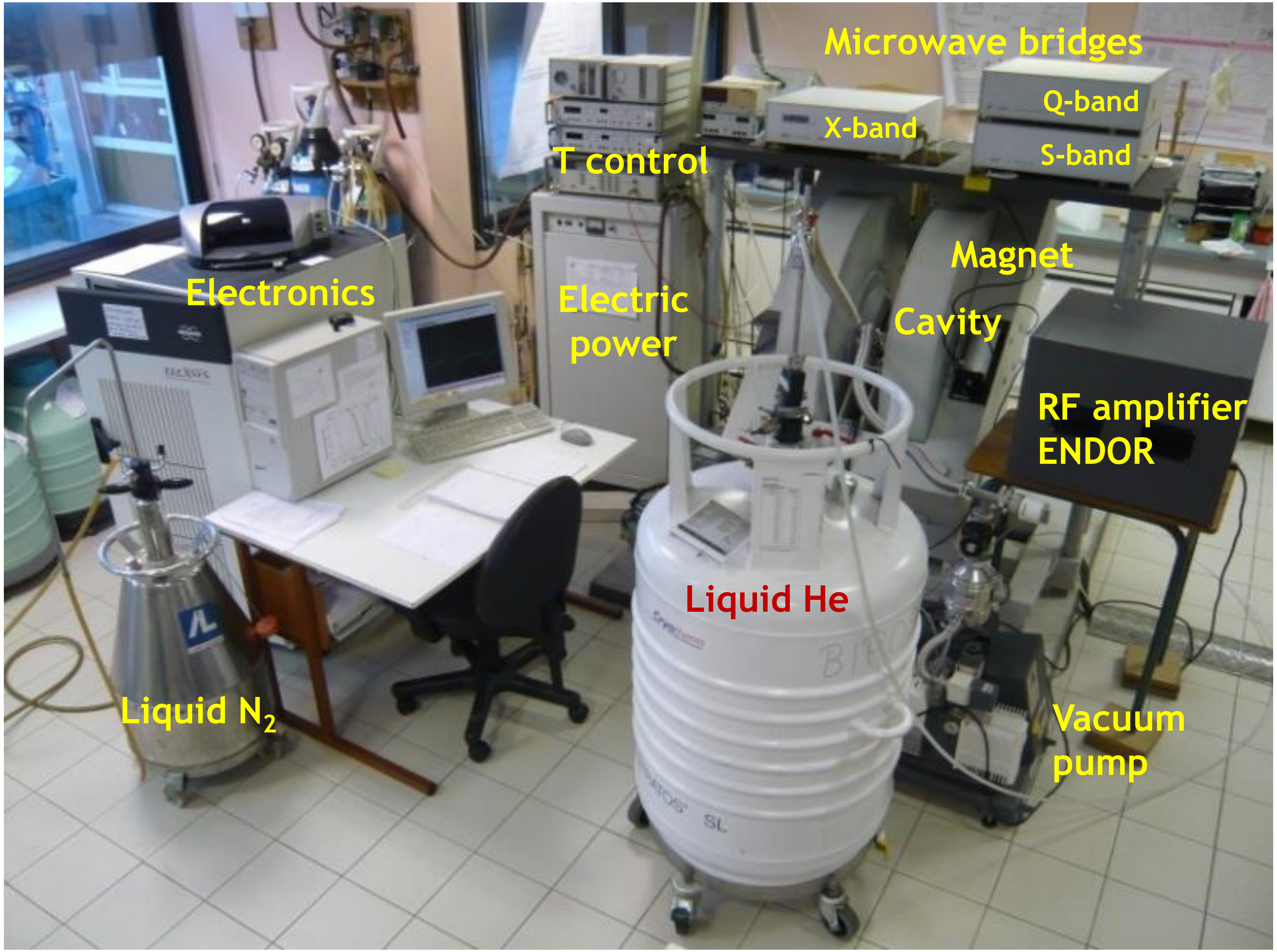
$b_m(t) = 1/2\ \Delta B_m \cos(2\pi\nu_m t + \varphi)$

ν_m = 100 kHz

Strong improvement of the Signal /Noise ratio

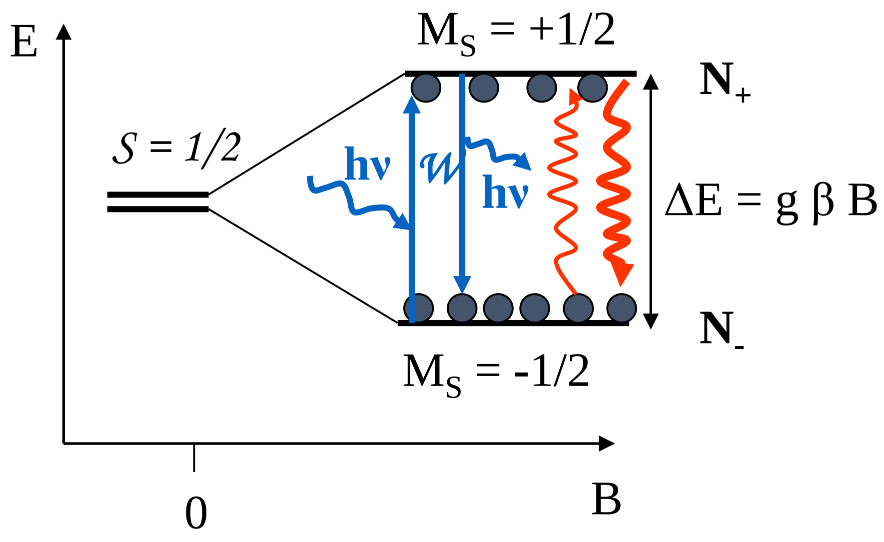
ΔB_m << linewidth δB (avoiding overmodulation)

Multifrequency CW-EPR equipment at BIP



MOSBRI
Molecular-Scale Biophysics
Research Infrastructure

Electron spin relaxation



Radiation induced transitions

Spontaneous transitions (relaxation):
- Spin-lattice relaxation, T_1
- Spin-spin relaxation, T_2

Upon microwave irradiation, competition between Absorption/Relaxation

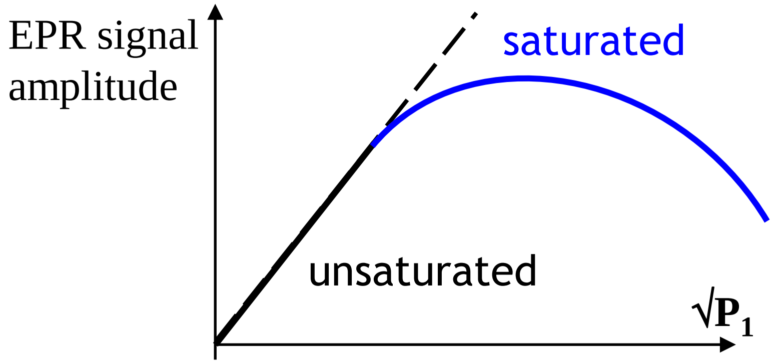
$$dn/dt = -2\,\mathcal{W}n + (n_0 - n)/T_1 \qquad (\mathcal{W} \propto B_1^2)$$

=> Steady state in continuous wave EPR

Net absorbed microwave power at steady state

$$P_{abs} = h\nu\,(\mathcal{W}N_- - \mathcal{W}N_+) = h\nu\,\mathcal{W}\,n_{Stat}$$

High power: $n_{stat} \rightarrow 0$: **Power saturation**



=> **T_1 and T_2 measurements**

Microwaves	absorption →	Spins	Heat ⇄	Lattice
B_1^2	$\mathcal{W}$	T_S	T_1	T

Electron spin relaxation: Temperature dependence

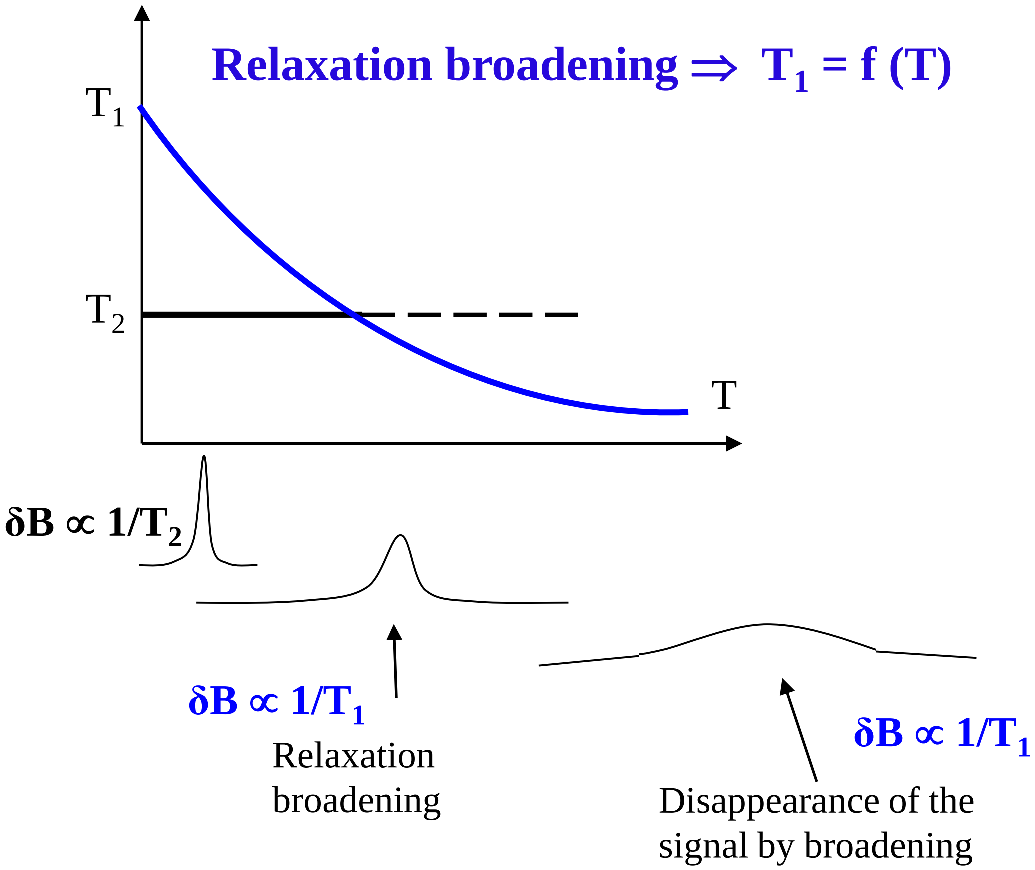
Spin-lattice relaxation (T_1):
- Coupling between spins and vibrations (phonons)
- Strong dependence on spin-orbit coupling

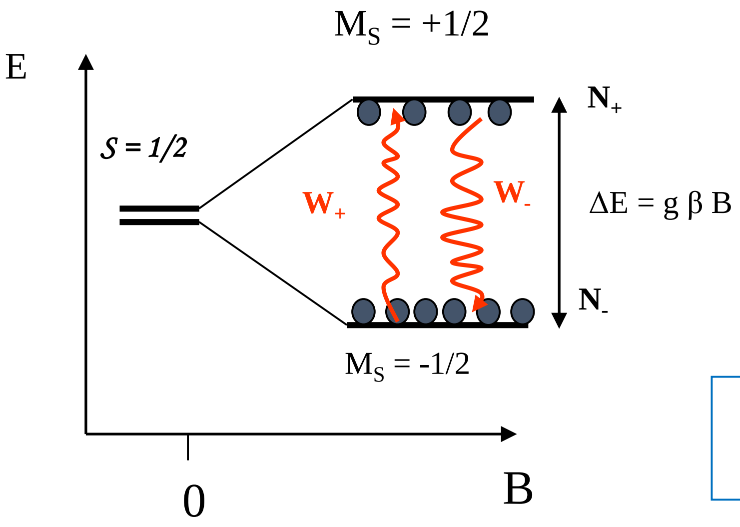
$$\mathbf{H_{SO} = \lambda\, L \cdot S}$$

- If T increases, T_1 decreases.

When $T_1 \approx T_2$ broadening of the resonance line:

$$\boldsymbol{\delta B = \hbar/g\beta \cdot 1/T_1}$$



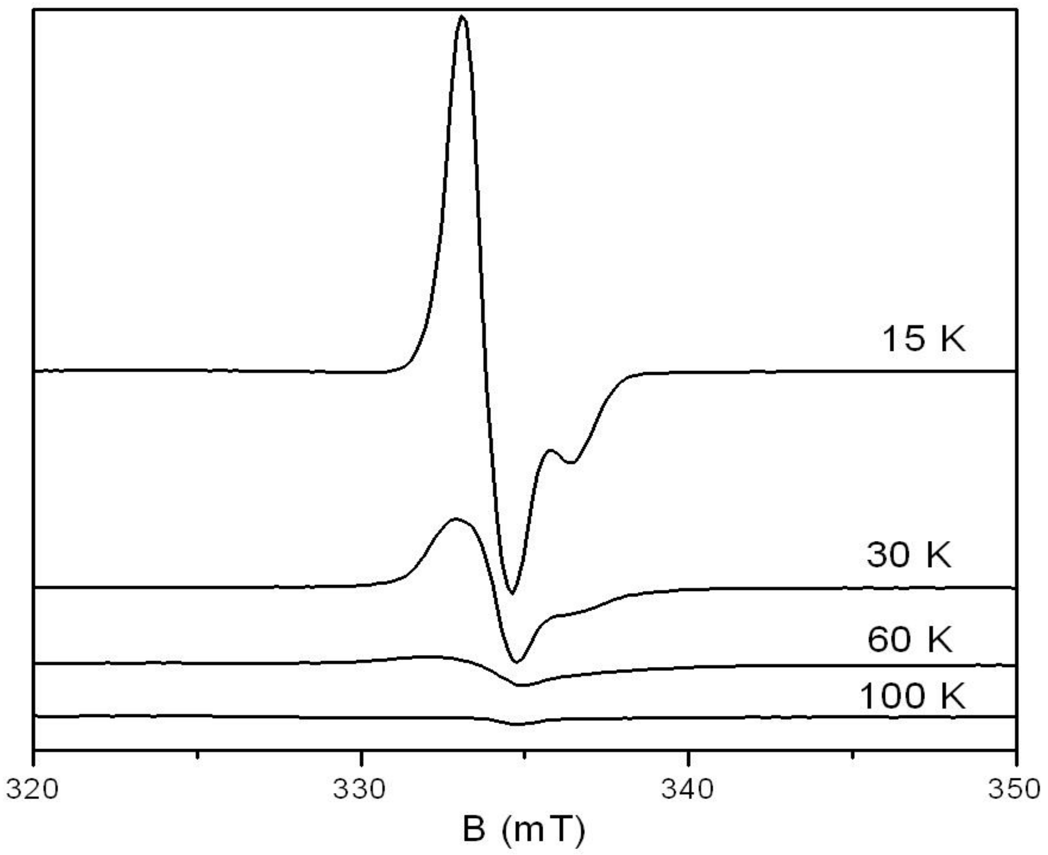


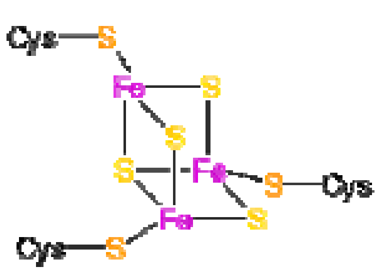
For transition metal ions : Strong spin-orbit coupling

- ***g*-tensor anisotropy**
- **Fast relaxation**
- **EPR study at low T**

Fe-S clusters: fast electron spin relaxation

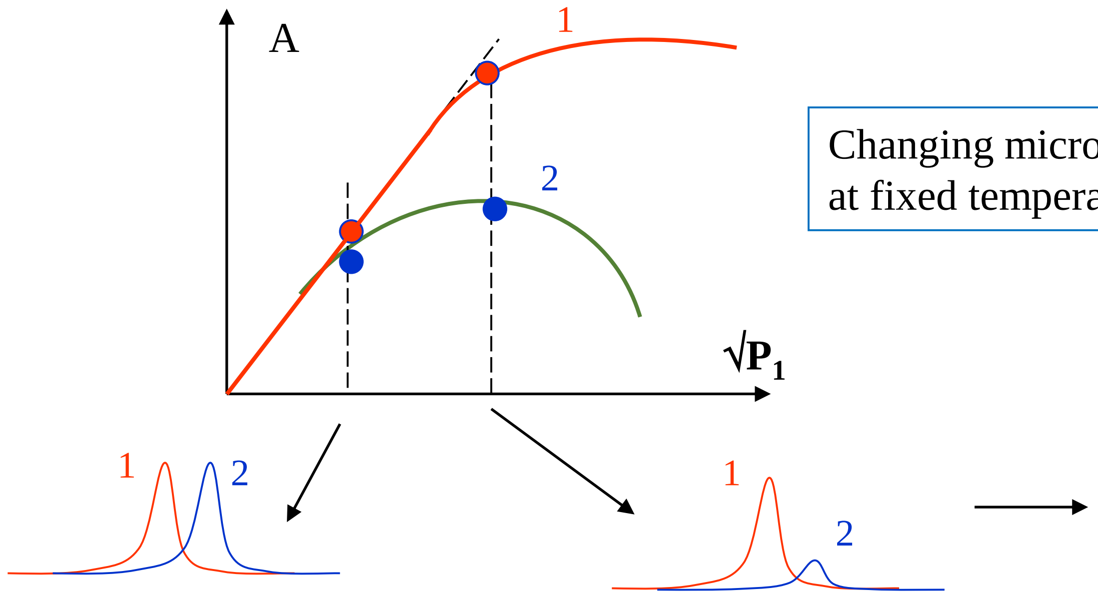
Relaxation broadening of a $[3Fe\text{-}4S]^{1+}$ signal (S = ½) upon T increase





Electron spin relaxation: Temperature dependence

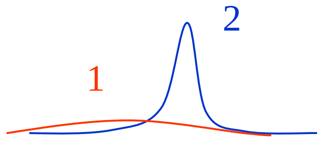
Strategies for separating signals from different species



Changing microwave power at fixed temperature

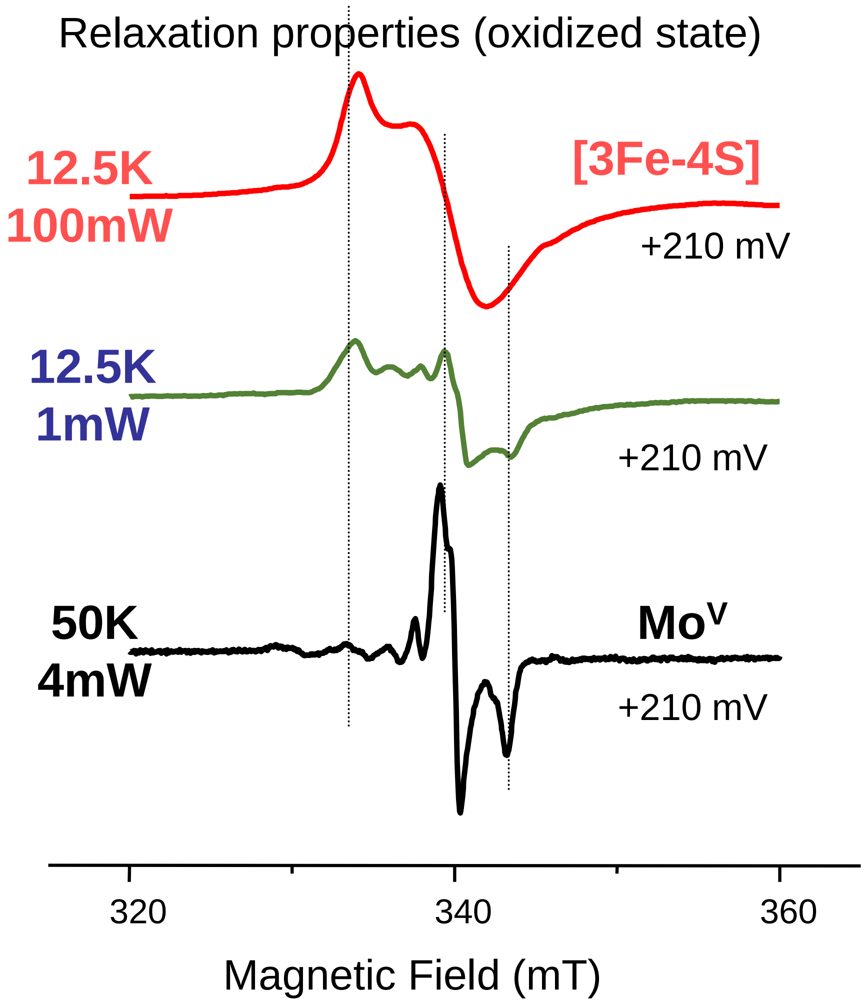
Separation of signals from species with different relaxation properties

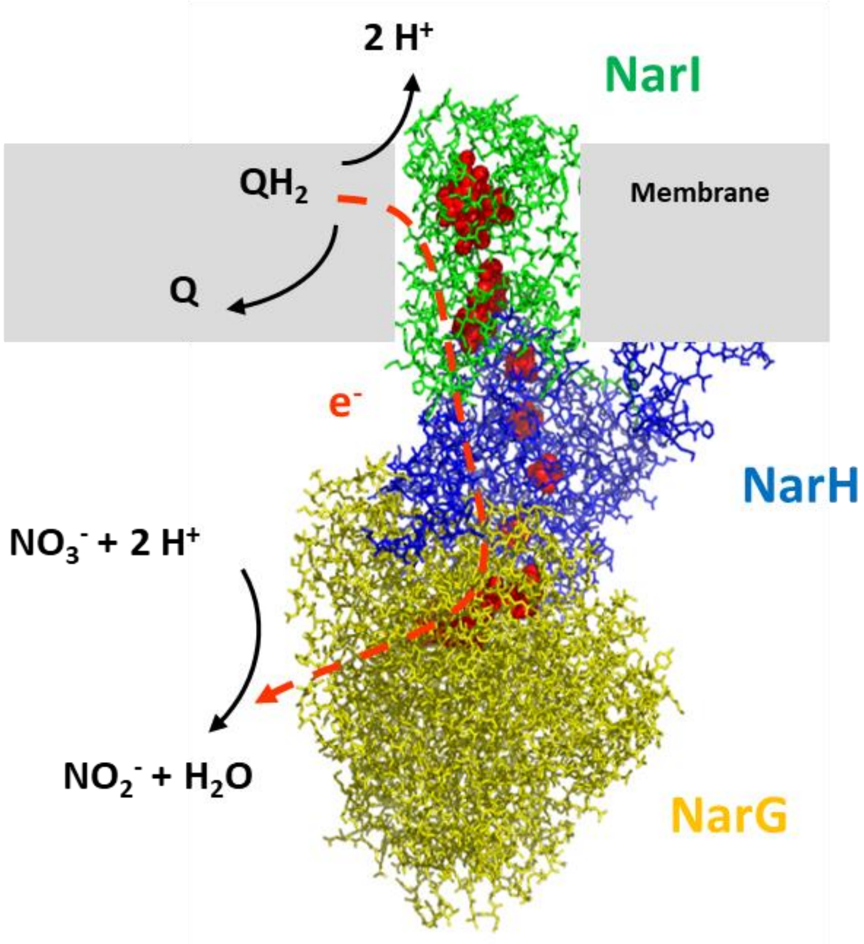
Increasing temperature to suppress a signal by relaxation broadening



Applications of EPR in the study of *E. coli* respiratory nitrate reductase

Selective EPR view of metal cofactors



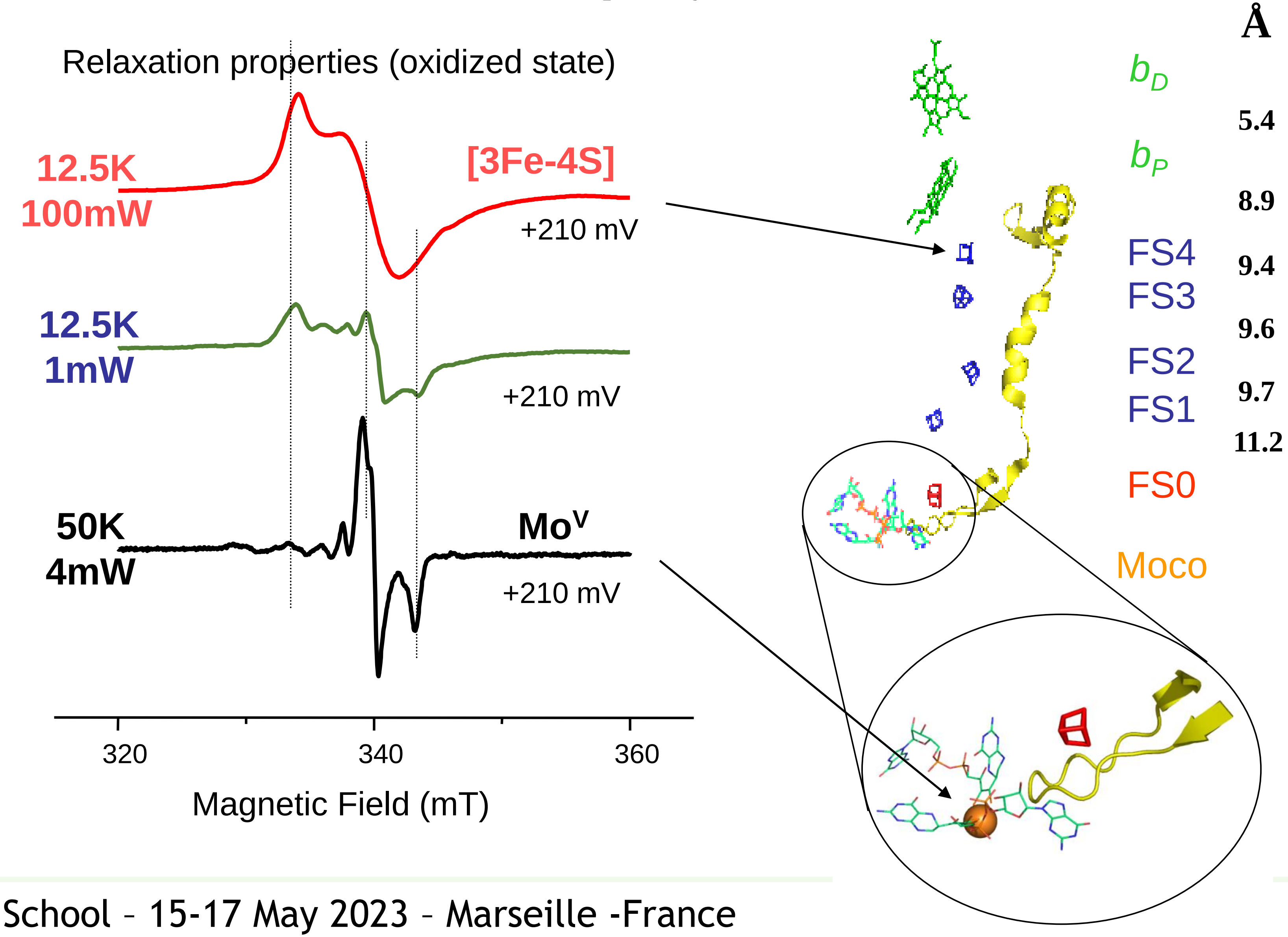


Membrane-bound Nitrate reductase from *E. coli* (NarGHI)

MOSBRI Molecular-Scale Biophysics Research Infrastructure

Applications of EPR in the study of *E. coli* respiratory nitrate reductase

Selective EPR view of metal cofactors in respiratory nitrate reductase




THANK YOU FOR
YOUR ATTENTION !