POLITECNICO DI MILANO

Department CMIC

Lecture 15 – FR15





# Free-Radicals: Chemistry and Biology

Prof. Attilio Citterio

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http://iscamap.chem.polimi.it/citterio/education/free-radical-chemistry/

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# Electron Paramagnetic Resonance (EPR):
# A. Instrumentation

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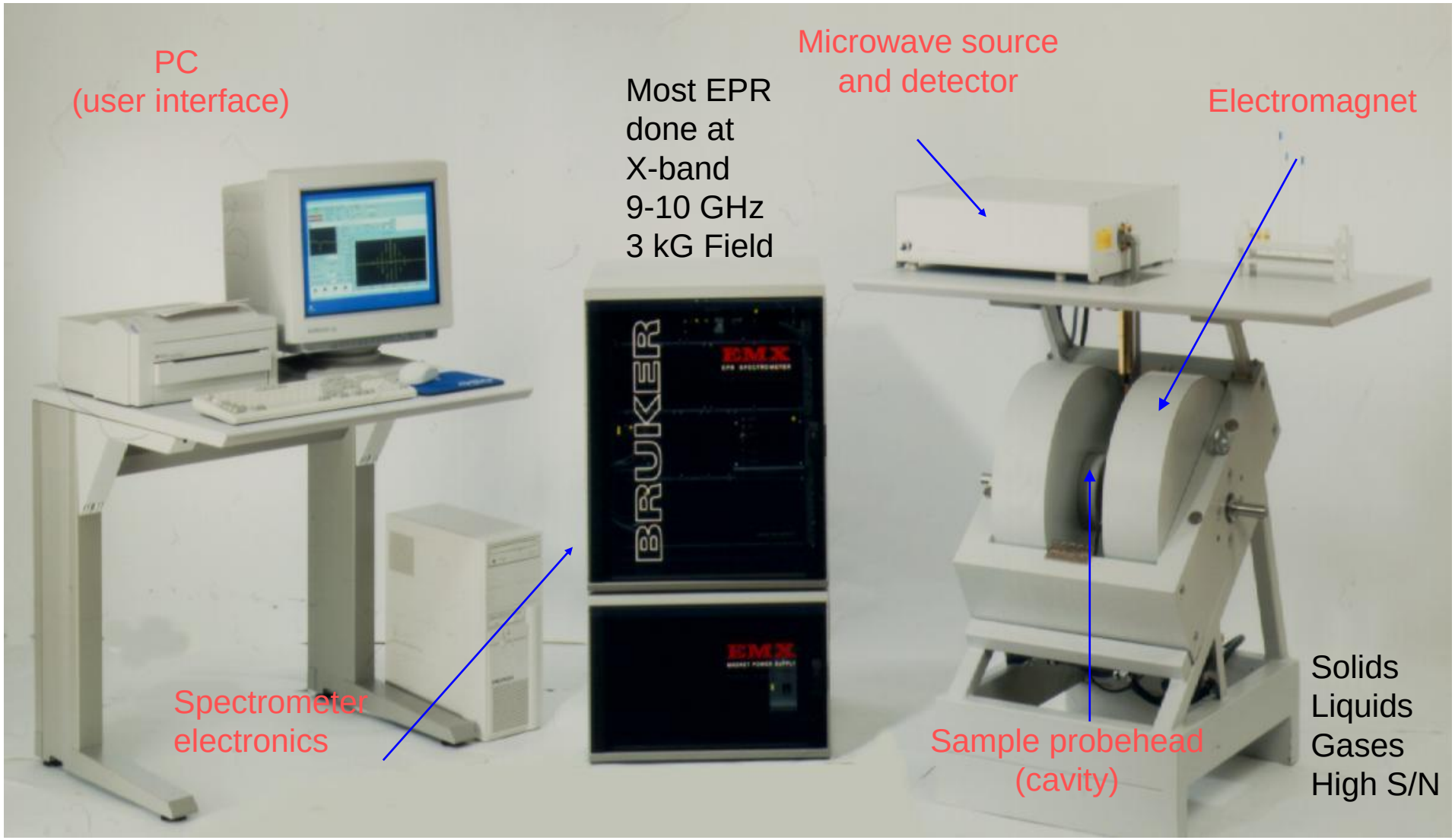
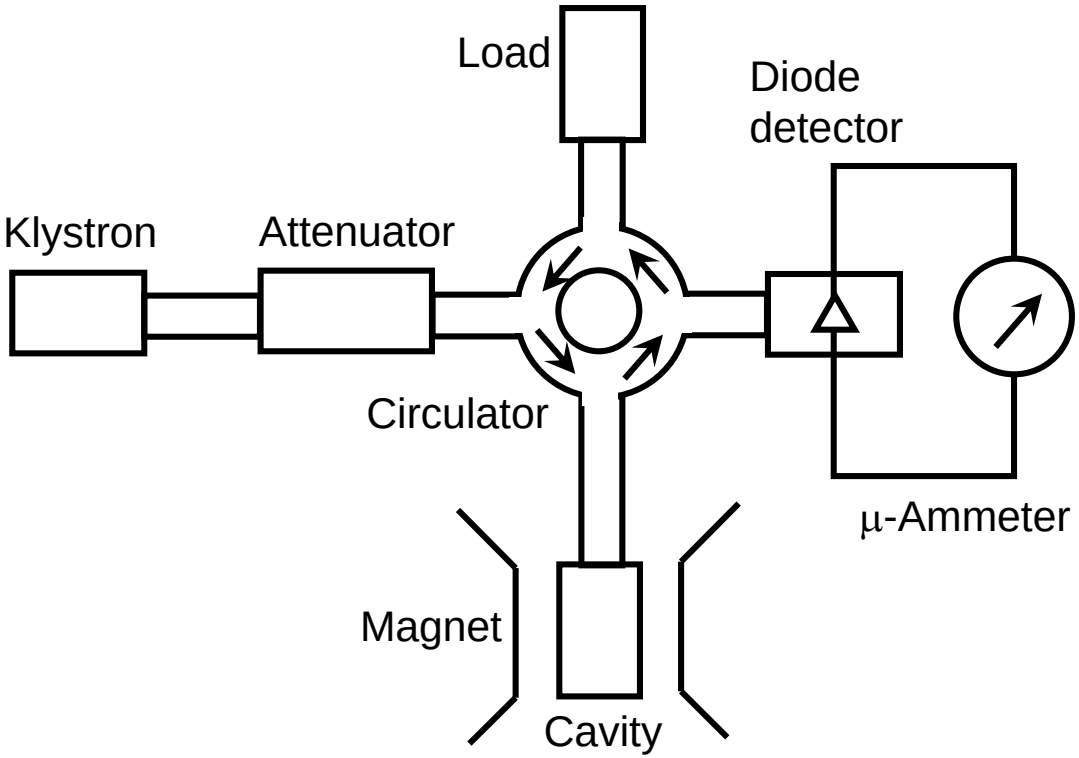
# Modern EPR Spectrometer



# Block Diagram of ESR Spectrometer



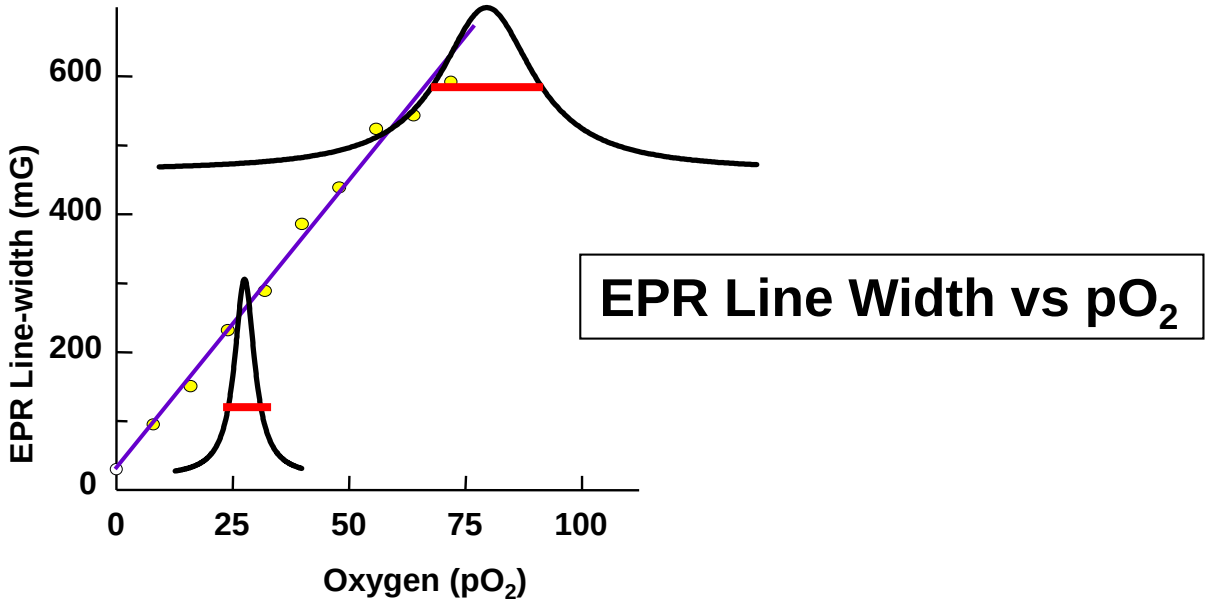

http://www.chm.bris.ac.uk/emr/Phil/Phil_1/p_1.html

# Application Techniques of Electron Spin Resonance

| Methods | Techniques | Applications |
|---|---|---|
| ▪ Direct ESR | ▪ Freeze Quench<br>▪ Snap Freeze<br>▪ Flat Cells<br>▪ AquaX<br>▪ Steady-State<br>▪ Fast-Flow<br>▪ Stopped-Flow<br>▪ Rapid Sampling<br>▪ Folch Extraction<br>▪ Bile Cannulation<br>▪ Other Techniques | ▪ *In Vivo* |
| ▪ Spin-Trapping | | ▪ *In Vitro*<br>▪ *In Situ* |

# Molecular Oxygen Provides Contrast to Paramagnetic Probes in EPRI

- Molecular oxygen is paramagnetic and provides contrast to paramagnetic probes.
- This causes spectral broadening (increase in line width)
- Line width changes from oxygen contrast > 200 % in EPR, In NMR such changes may be ~10%




It is possible to image spatial distribution of paramagnetic spin probe by EPR and obtain $pO_2$ information

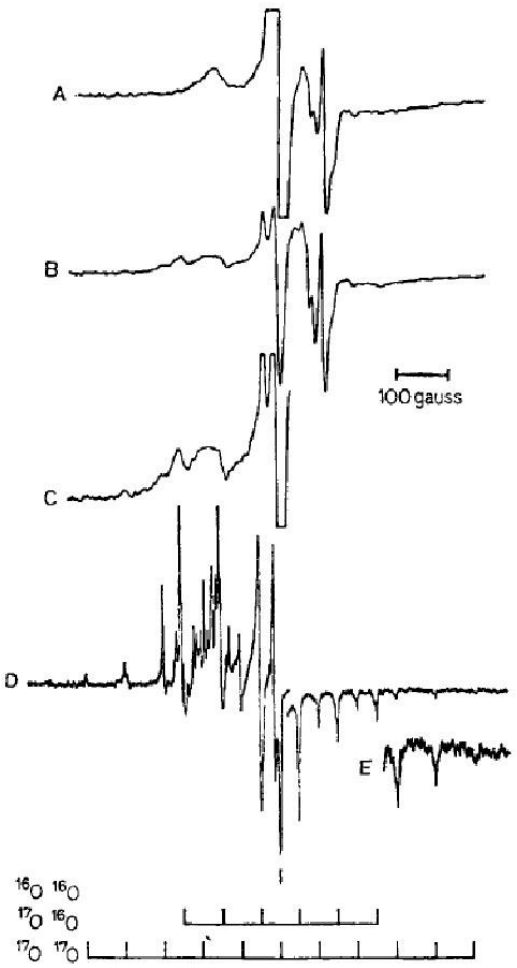
# Direct ESR

**"Freeze" the reaction**

1) **freeze quench *(in vitro)***
2) **snap freeze (*in vitro, ex vivo*)**

**Steady-State**

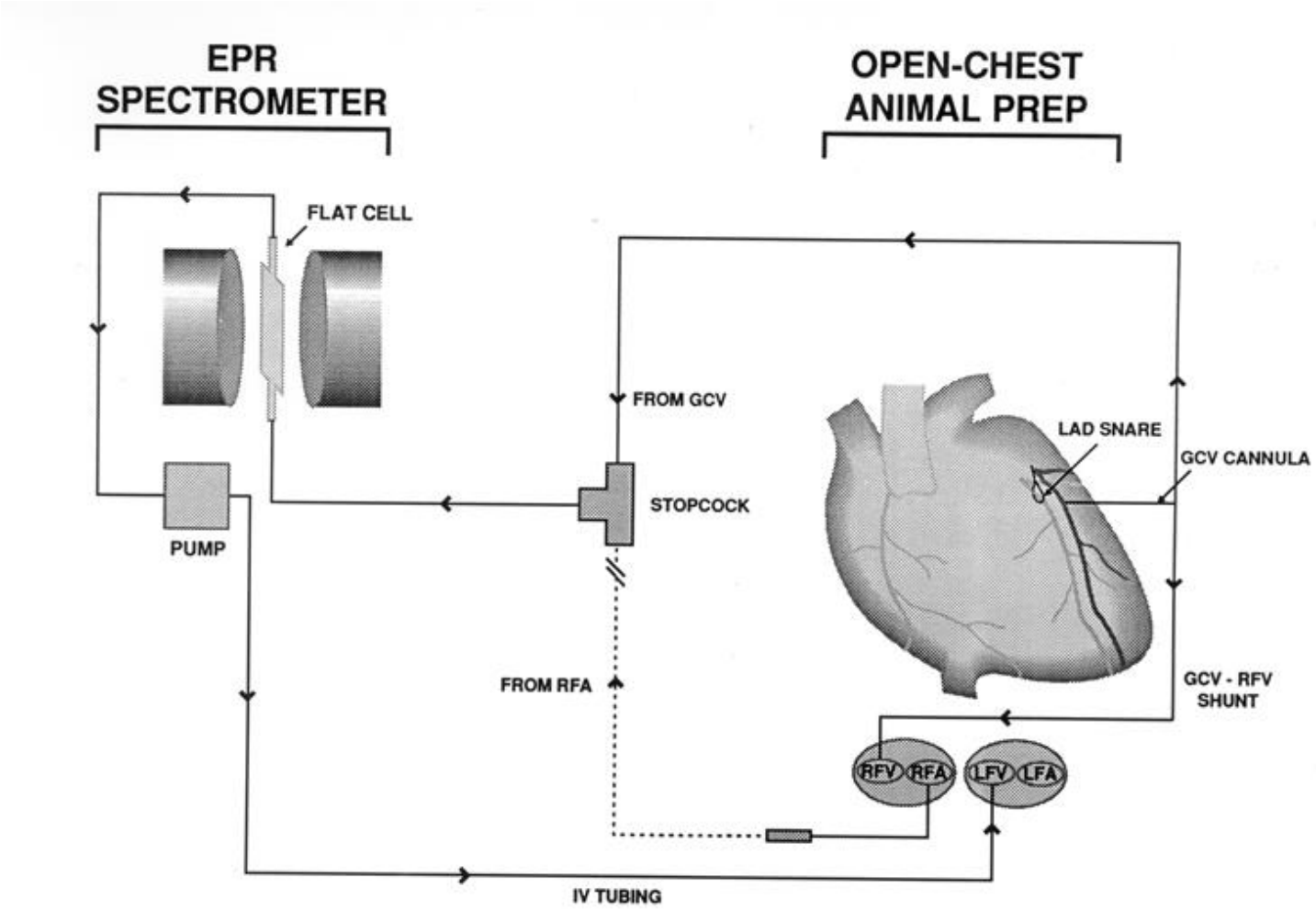
1) **Rapid sampling *(in vitro )***
2) **Fast-flow *(in vitro)***



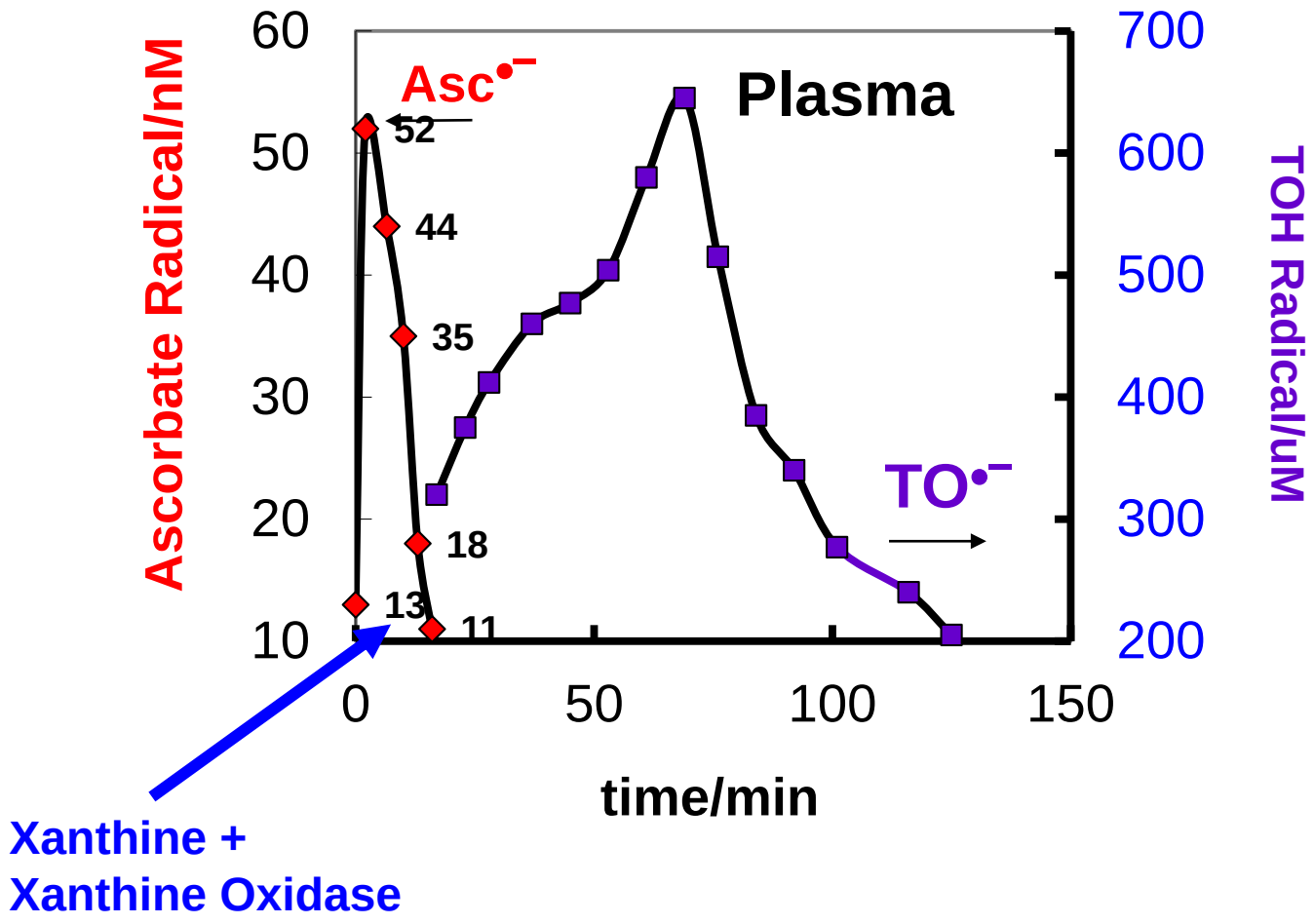

**The electron paramagnetic resonance spectrum of $^{17}O$ in $O_2^{\bullet -}$ generated during steady-state oxidation of xanthine catalyzed by xanthine oxidase. Both the 11-line spectrum from $^{17}O^{17}O^{\bullet -}$ and the six-line spectrum from $^{17}O^{16}O^{\bullet -}$ were detected. The results provide final confirmation that one-electron reduction of oxygen can occur in biological systems.**

**Bray, R.C., Pick, F.M. and Samuel, D., Eur J. Biochem, 15 352-355, 1970**

# Whole Animal Studies by ESR



# $AscH^-$ Recycles Tocopherol




Sharma MK, Buettner GR. *Free Rad Biol Med* **1993**, *14*, 649-653.

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# ESR Detection of Radicals:
# B. Direct Detection

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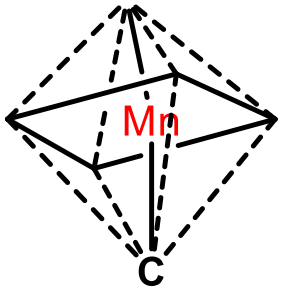
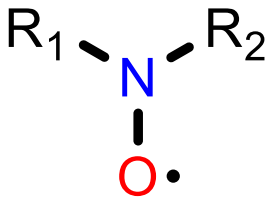
# Electron *Paramagnetic* Resonance
# Electron Spin Resonance

A spectroscopic technique to detect all paramagnetic species:

- *Persistent paramagnetic species*
- *Transient paramagnetic species*

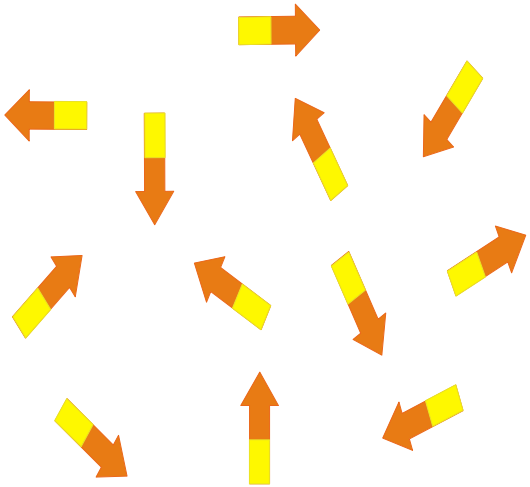

Free Radicals
Ascorbate
Hydroxyl
etc.

Transition Metals
All that have unpaired electrons

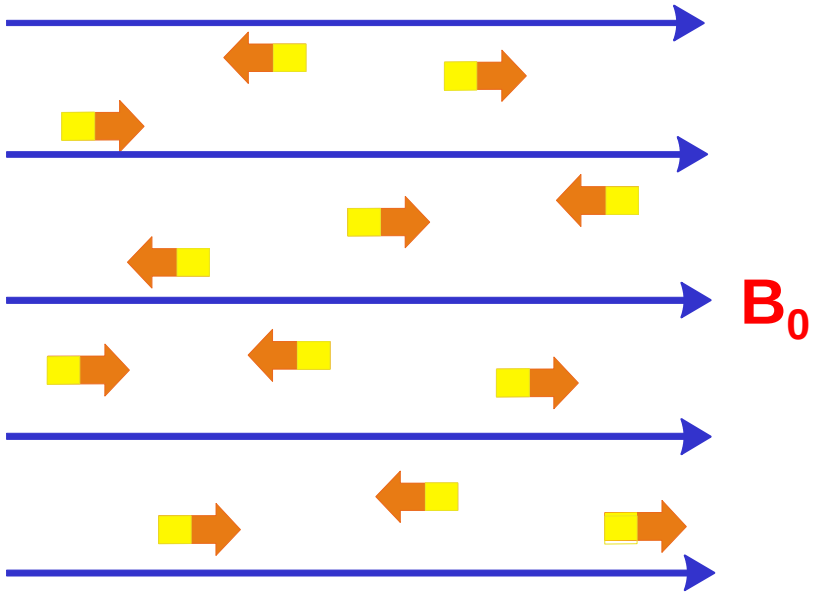
Spin Labels
$R_1$ & $R_2$ chosen to provide specificity

Spin Traps

# Alignment of Spin Moments in the Presence of a Magnetic Field.

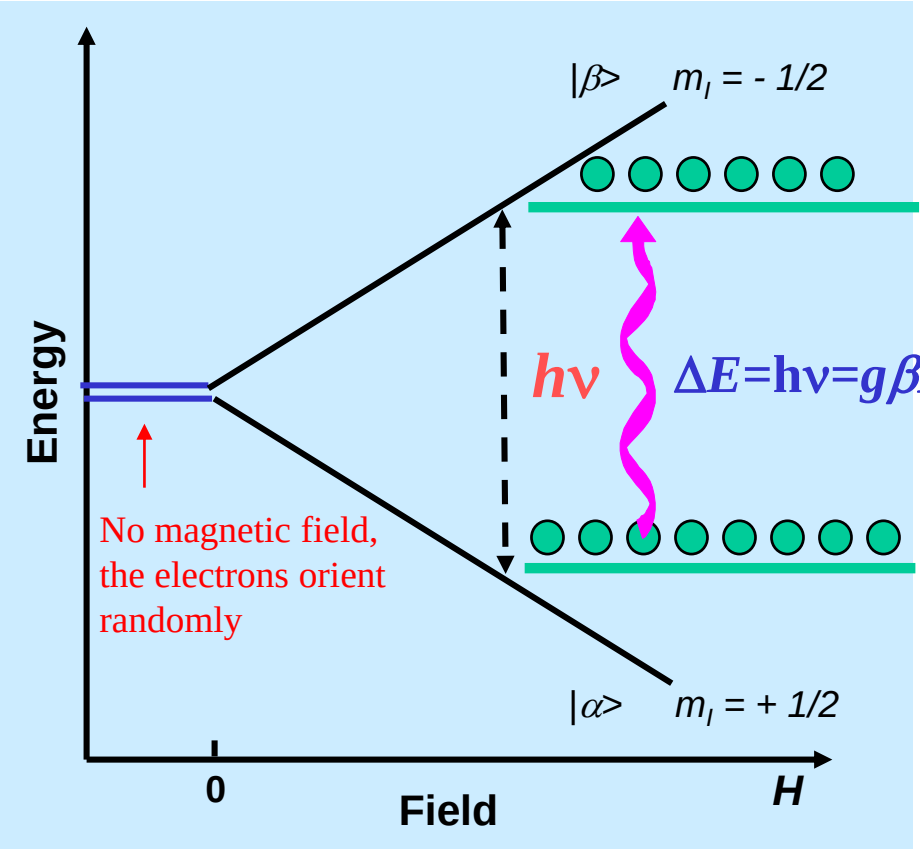


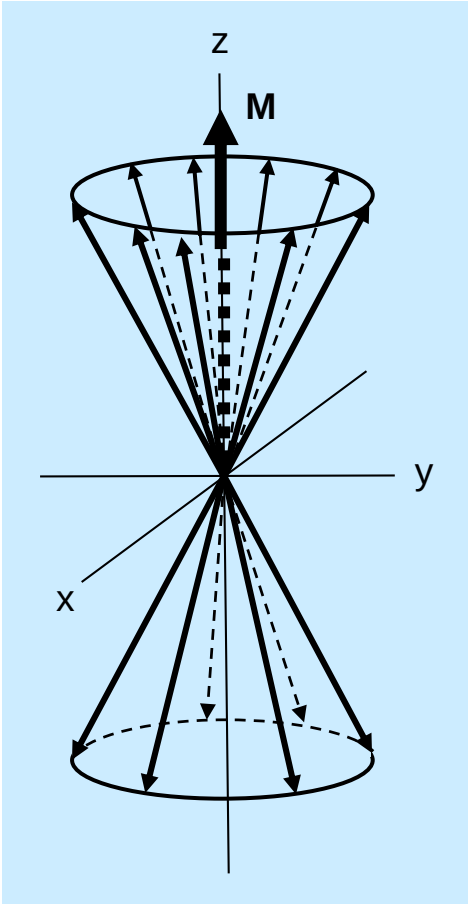
In absence of an external magnetic field the electron's magnetic moment will orient randomly.




When a larger external magnetic field is applied, the electrons will align either with or against this field.

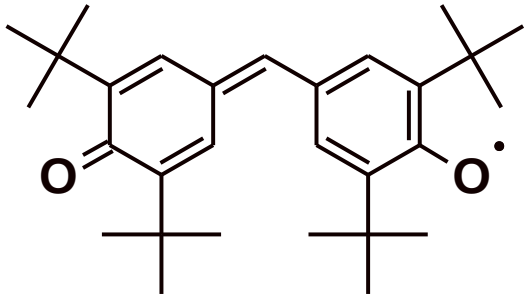
# Spin ½ Electron in an External Field: Zeeman Effect

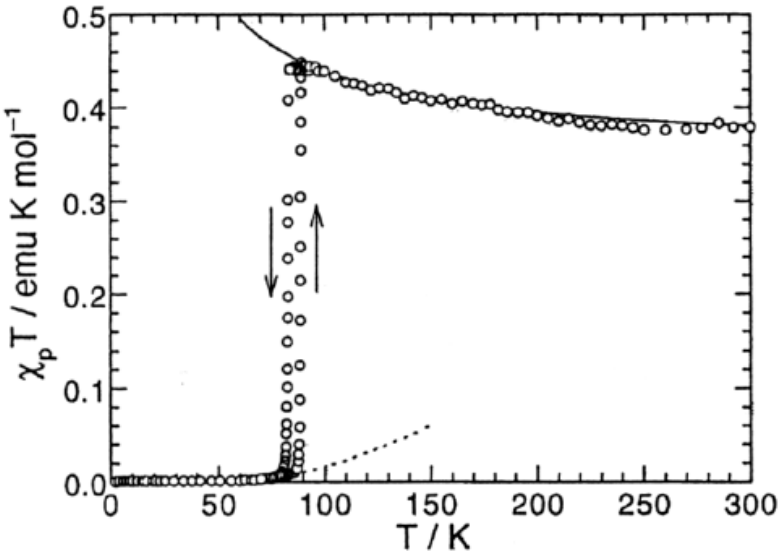


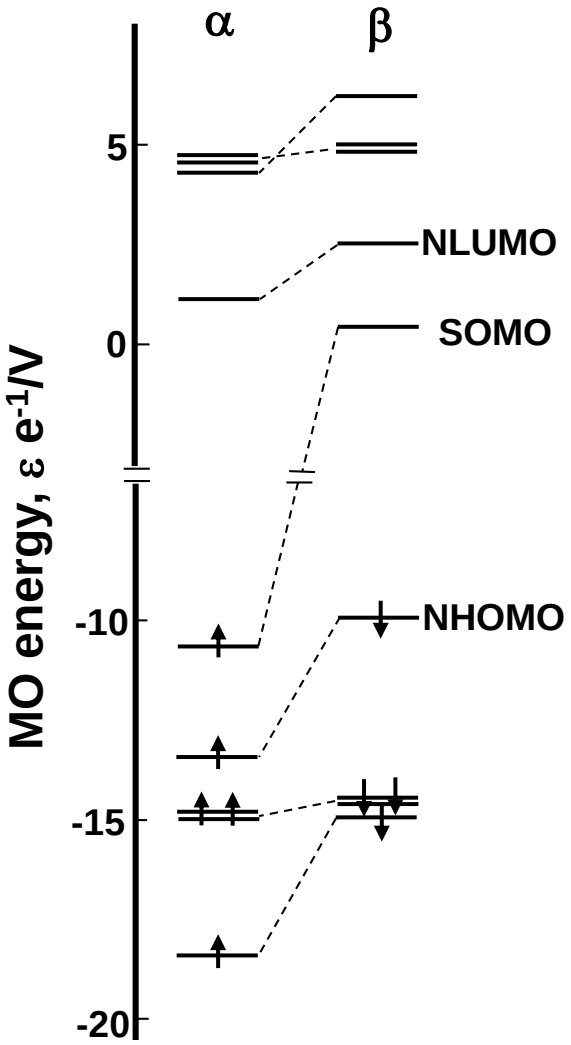




Electron Spins Precess at the Larmor Frequency about the External Field $\gamma_e$ = 1.76e$^7$ rad/sec/G

# Temperature Effect: Galvinoxyl

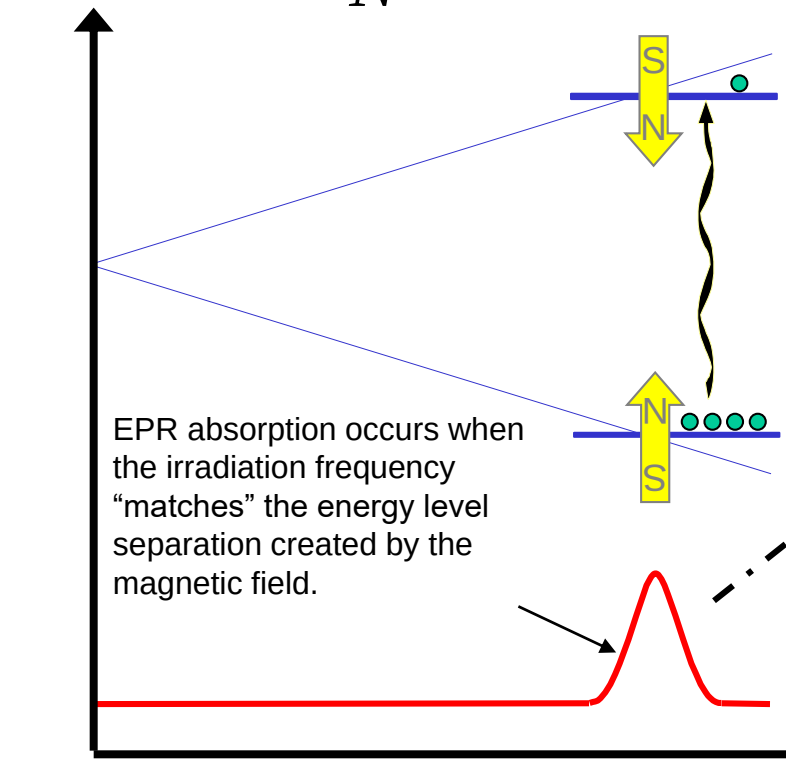


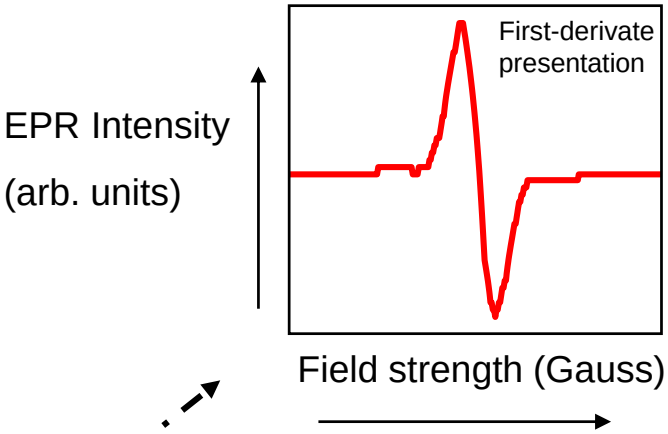



# EPR Spectrum

$$\frac{N^+}{N^-} = e^{-\frac{h\nu}{kT}}$$




The EPR signal is processed and output as a 1st derivative of the absorption spectrum.

Note that the width between points of maximum slope ($\Delta H_{MS}\alpha$) is easily measured and is the standard representation of band width.

Typically, $\nu_0$ = *ca.* 9.2 × $10^9$ Hz (9.2 GHz)
(i.e. $\lambda_0$ = *ca.* 3 cm, which is X-band microwave radiation)
If g = 2, then $B_0$ will be ca. 0.33 T (3300 G)

# Magnetic Field Effect

**Divergence of the ±1/2 levels of an electron in a radical as the external magnetic field (B) is increased.**

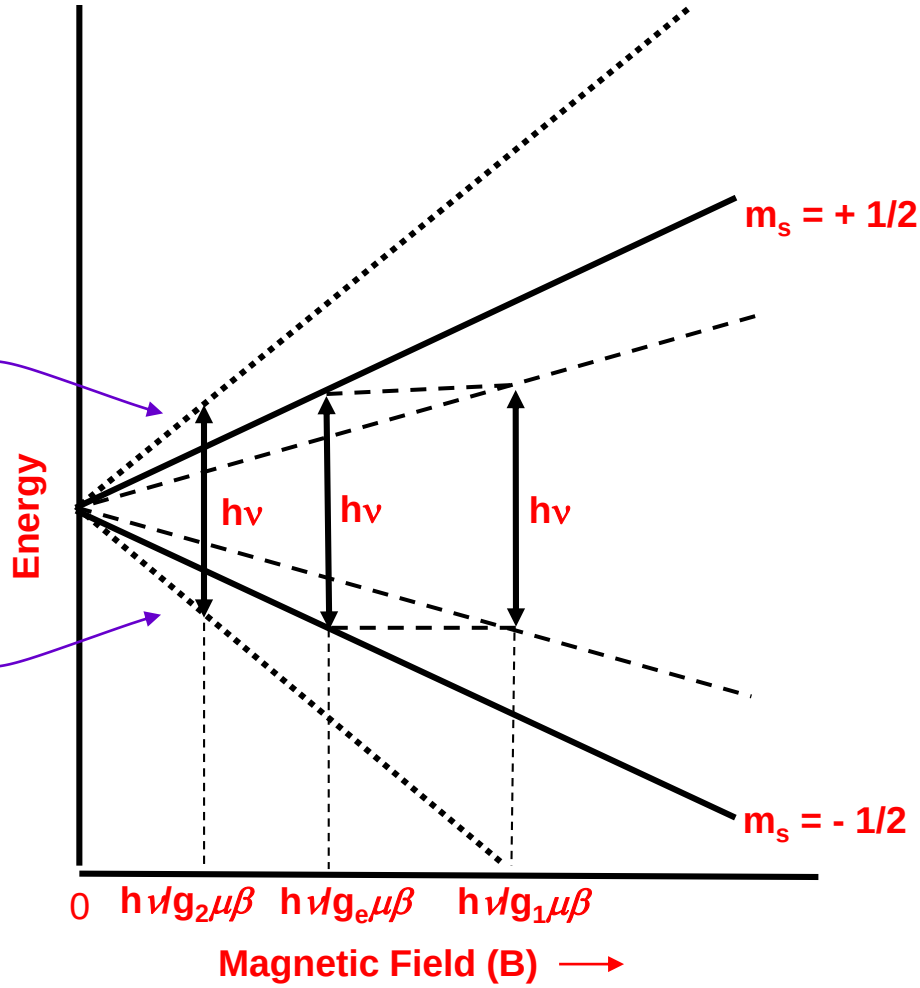
$N_{+1/2}$ = ca. $4.996 \times 10^{13}$ (300 K)

$N_{-1/2}$ = ca. $5.004 \times 10^{13}$ (300 K)

(if $10^{14}$ radicals in the sample)

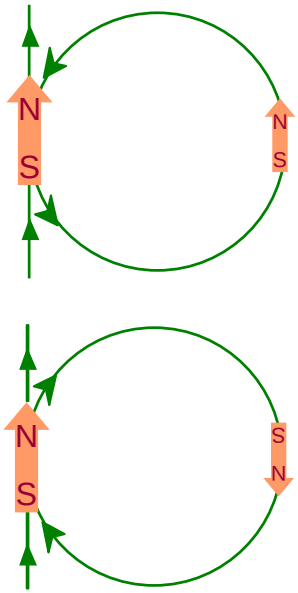
$N_{-1/2}/ N_{+1/2} = e^{(h\nu_0/kT)}$ = 1.0015 (300 K)

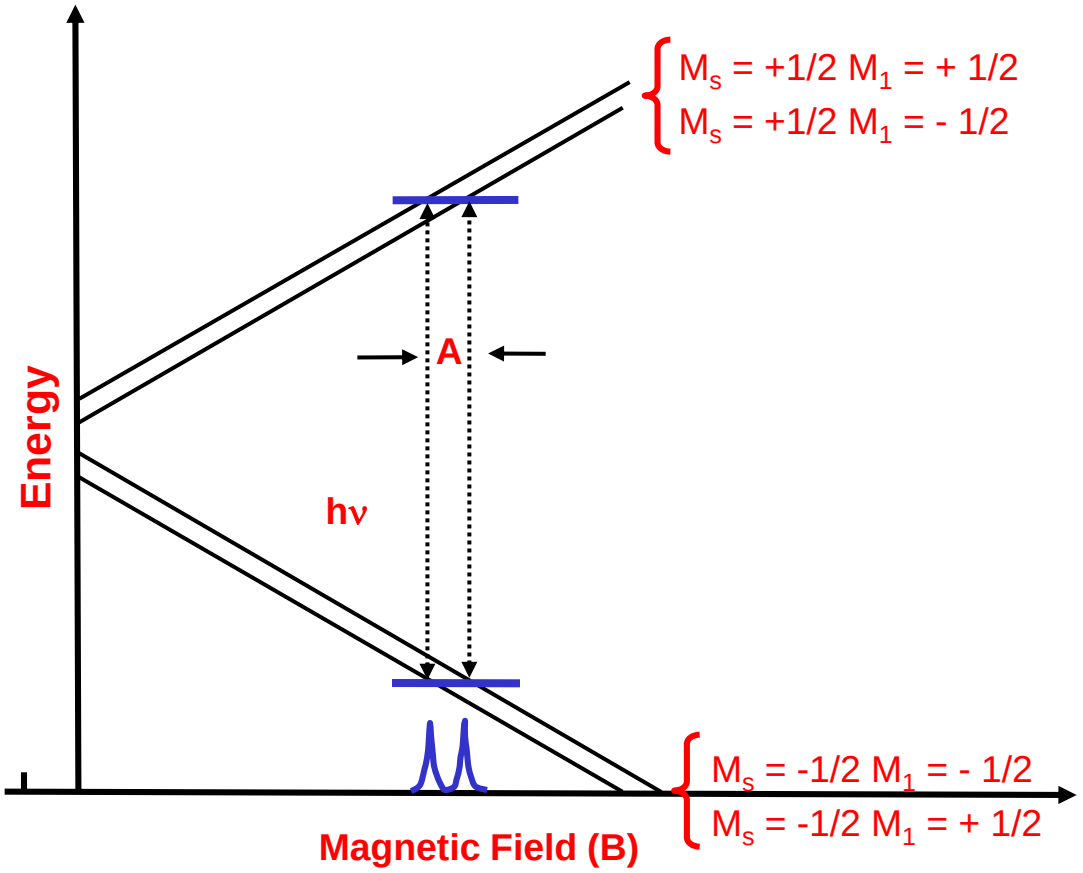
= 1.0044 (100 K)



# EPR Hyperfine Coupling

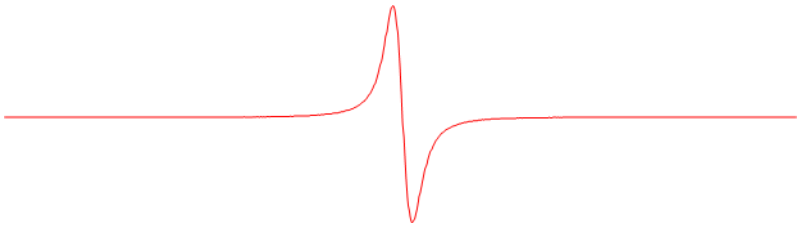
**Effect of hyperfine coupling to a single nucleus with *I* = ½ in the high-field region.**



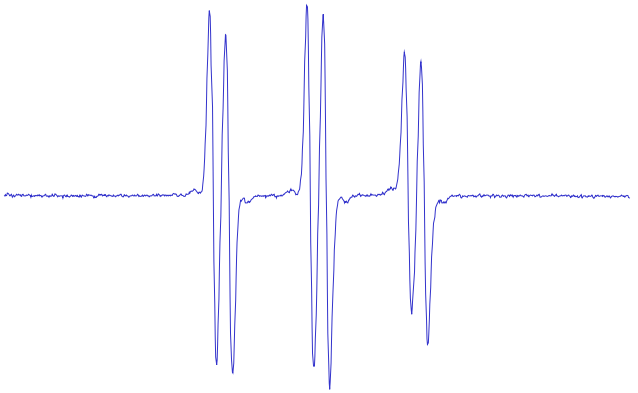



# Hyperfine Splitting

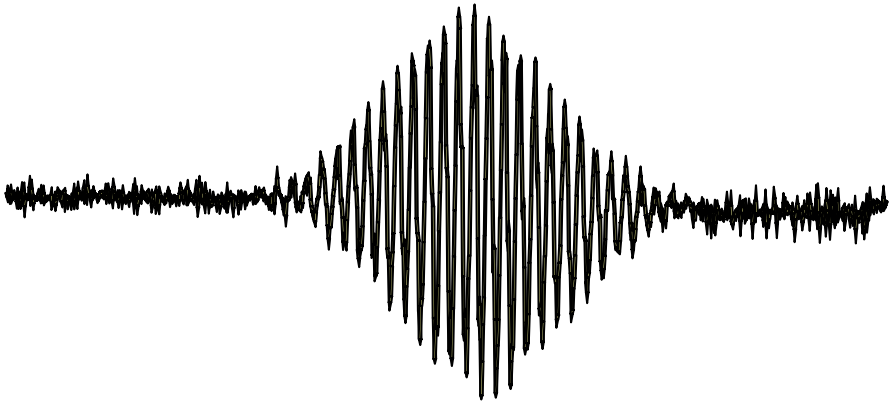
No hyperfine interaction

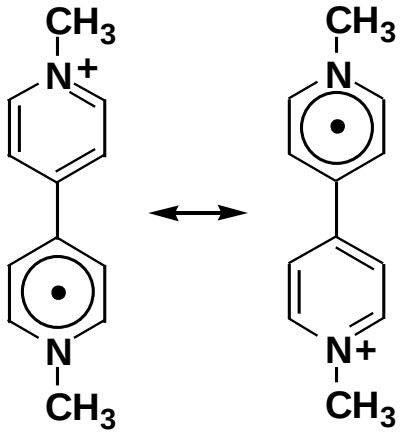


Simple hyperfine interaction



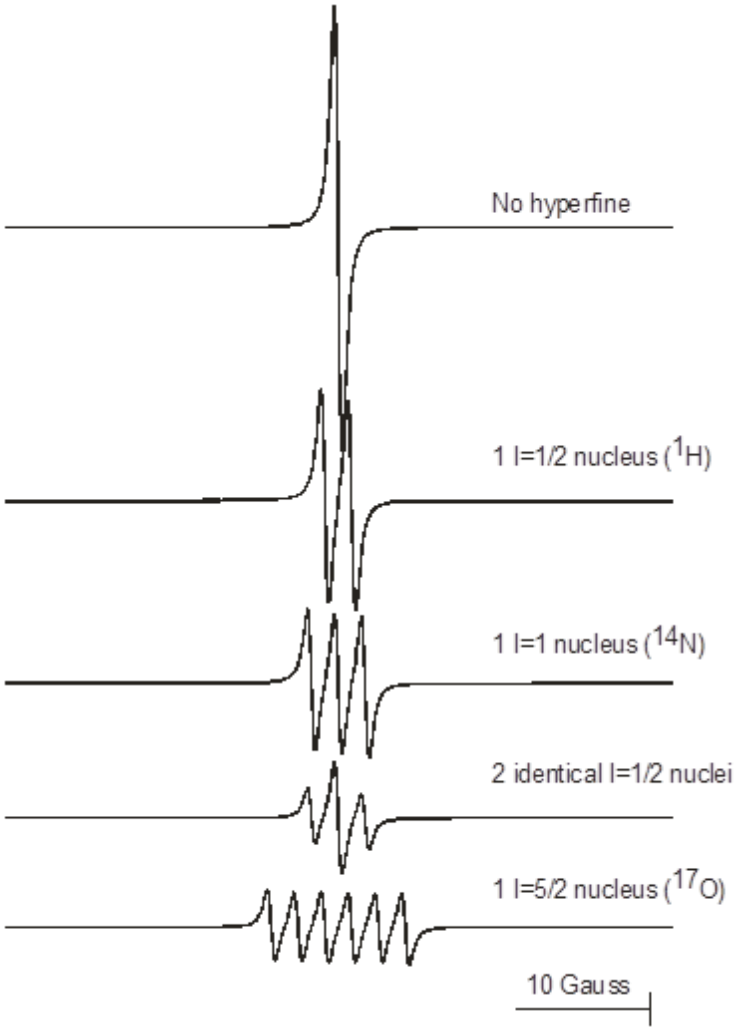
Multiple hyperfine interactions



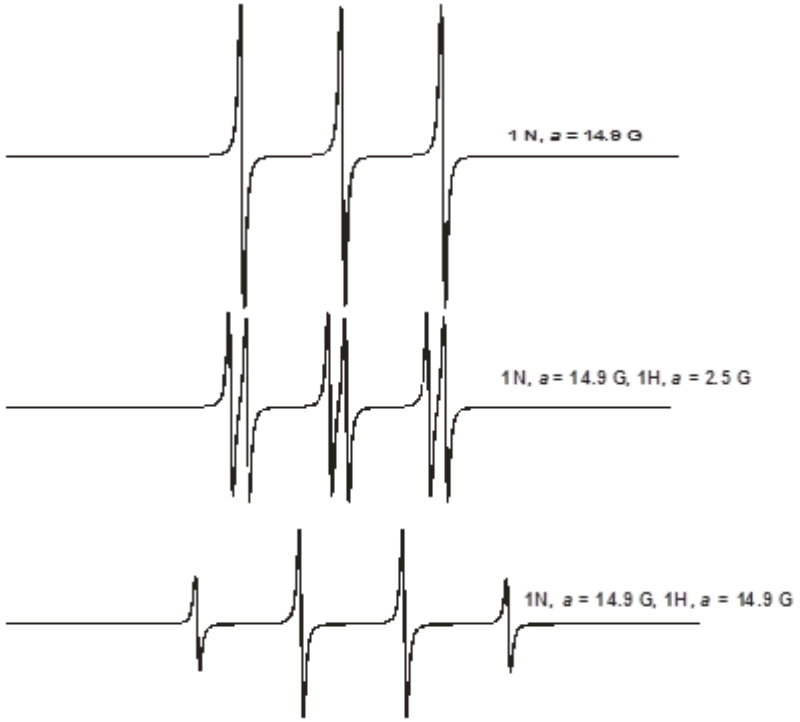


# Number of Lines and Magnetic Moment of Nuclei

**# of lines = 2n(I + ½),** so I = ½ gives 2 lines, I = 1 gives 3 lines, etc.




Hyperfine splittings are additive



# The g-Factor

**EPR: g-factor**

$$g = \frac{h\nu}{\beta B_0}$$

$h$ = Planck's constant

$\nu$ = Microwave frequency

$\beta$ = Bohr magneton

$B_0$ = Magnetic field

| Microwave Band | Microwave Frequency (GHz) | $B_0$ (for g=2) Gauss |
|---|---|---|
| L | 1.1 | 392 |
| S | 3.0 | 1070 |
| X | 9.5 | 3389 |
| K | 24.0 | 8560 |
| Q | 35.0 | 12485 |
| W | 94.0 | 33600 |

# g-factor

$$g = g_{spin\ only} + \delta g = 2.0023 + \delta g$$

1. **The MAGNITUDE** of $\delta g$ depends on the size of $(\zeta/\Delta)$; the major variation ia in $\zeta$

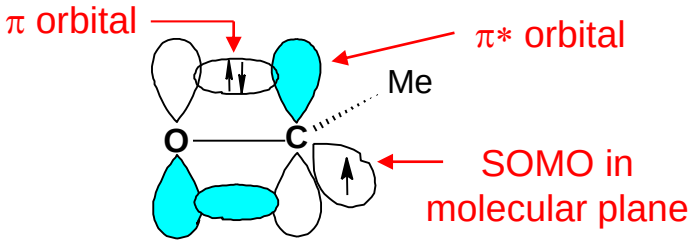
| Atom | C | N | O | F | Cl | Br |
|---|---|---|---|---|---|---|
| Z | 6 | 7 | 8 | 9 | 17 | 35 |
| $(\zeta/cm^{-1})$ | 29 | 76 | 151 | 270 | 586 | 2460 |

2. **The SIGN** of $\delta g$ depends on the detailed electronic configuration and orbital energies of the radical. For most types of organic radicals $\delta g$ is **positive**, although it can be **negative** especially for certain $\sigma$ radicals e.g. acyl radicals.

   **Simplified view:** orbital magnetism restored by the unpaired electron moving via:

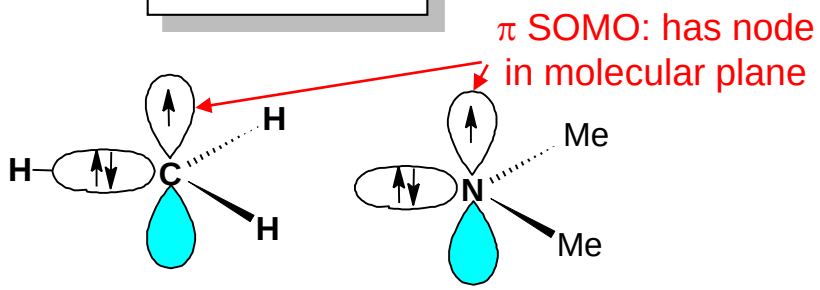
   (a) Originally **filled** orbitals, $\delta g$ + ve (b) Originally **empty** orbitals, $\delta g$-ve

$\sigma$ RADICAL




SOMO (USUALLY) HAS SIGNIFICANT s-A.O. CARACTER

$\pi$ RADICAL




SOMO HAS (ESSENTIALLY) NO s-A.O. CARACTER

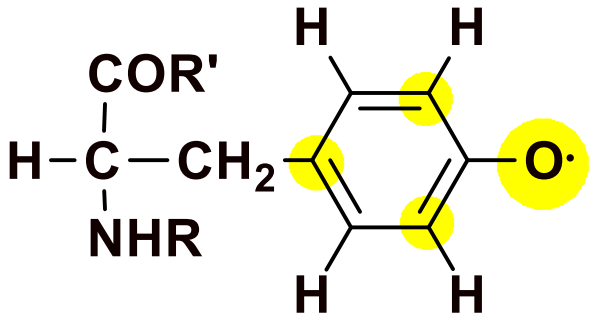
# ΔE = gβH g-factor ranges for several classes of free radicals*

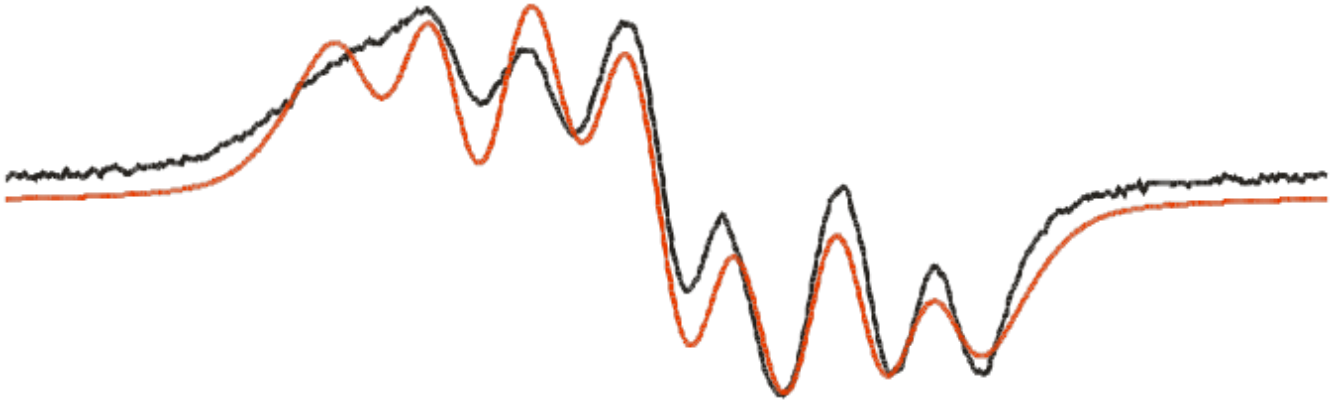
| class | Range of typical *g* |
|---|---|
| Chlorophyll and related porphyrin cations and anions; polycyclic hydrocarbon cations / anions | 2.0024 ~ 2.0028 [b] |
| Flavosemiquinones | 2.0030 ~ 2.0040 |
| Benzosemiquinones, aryloxy, and phenoxy radical ions | 2.0040 ~ 2.0050 |
| Nitroxides | 2.0050 ~ 2.0060 |
| Peroxyl radicals | 2.01 ~ 2.02 |
| Sulfur-containing radicals | 2.02 ~ 2.06 |

[a] Data from Bolton [8]

[b] Values as low as 2.000 (zinc tetraphenylporphyrin anion, $ZnTPP^-$) and as high as 2.004 ($ZnTTP^+$ $Br^-$) are known, however.

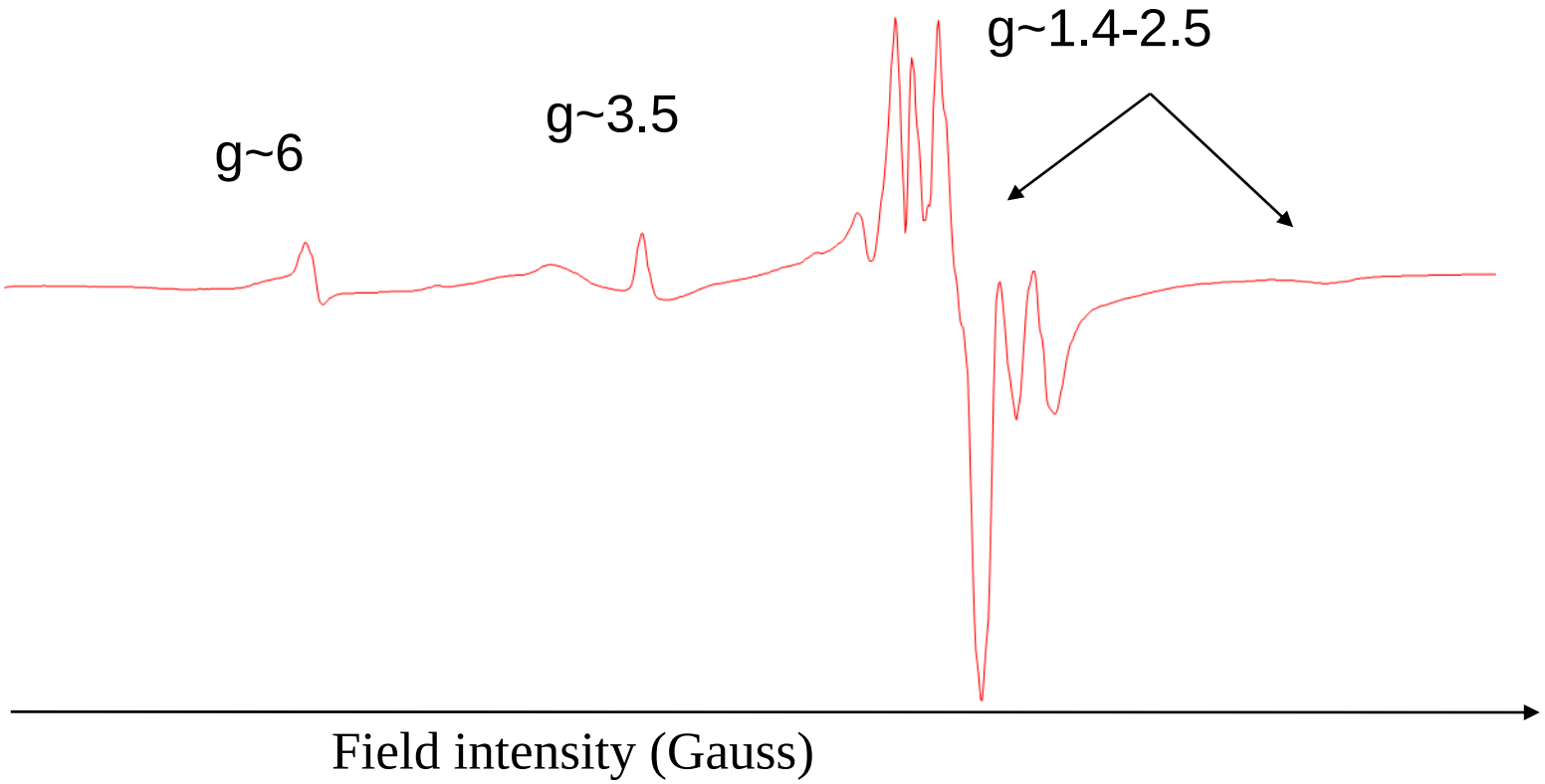
# Direct EPR of a Tyrosyl Radical



Gunther, M.R., Sturgeon, B.E., and Mason, R.P., *Free Radic. Biol. Med*. **2000**, *28*, 709-719,

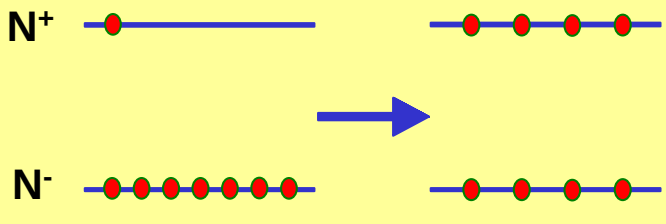
# g-Factor in Metalloproteins




Cytochrome oxidase is a metalloprotein with more than one metal center. The g-values are used to identify and characterize the different centers.

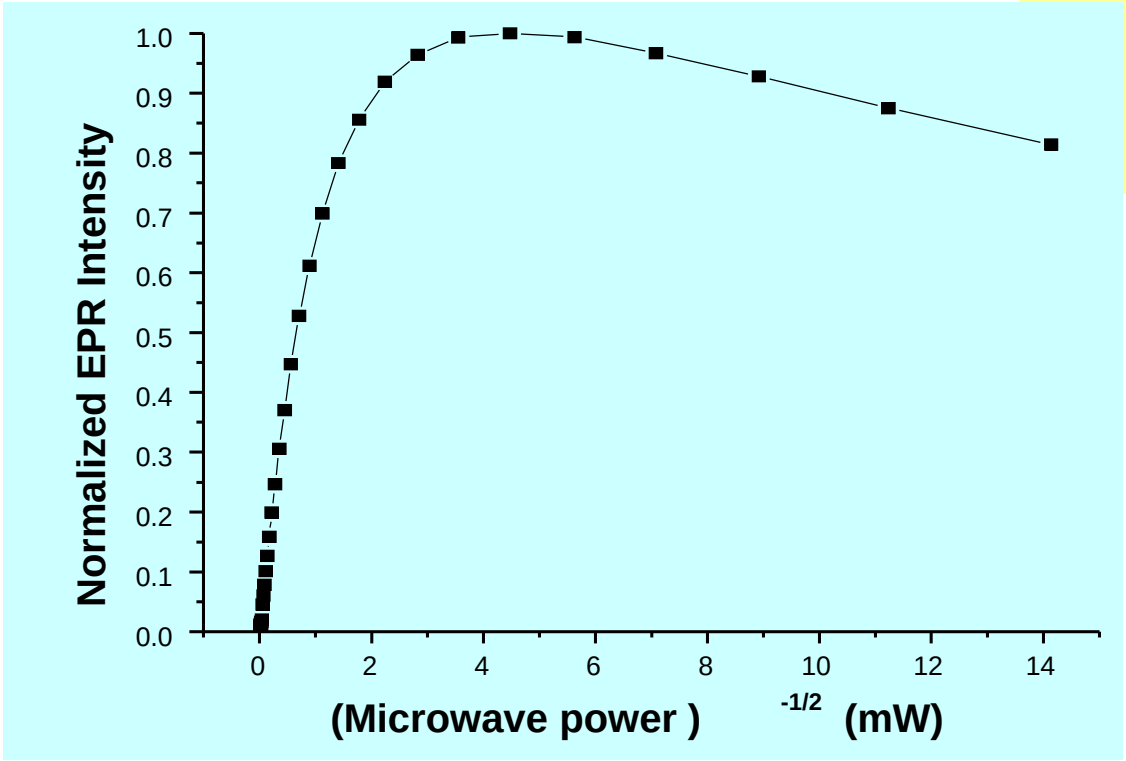
# Saturation of EPR Signal

In EPR under saturation $\frac{N^+}{N^-} = 0.995$

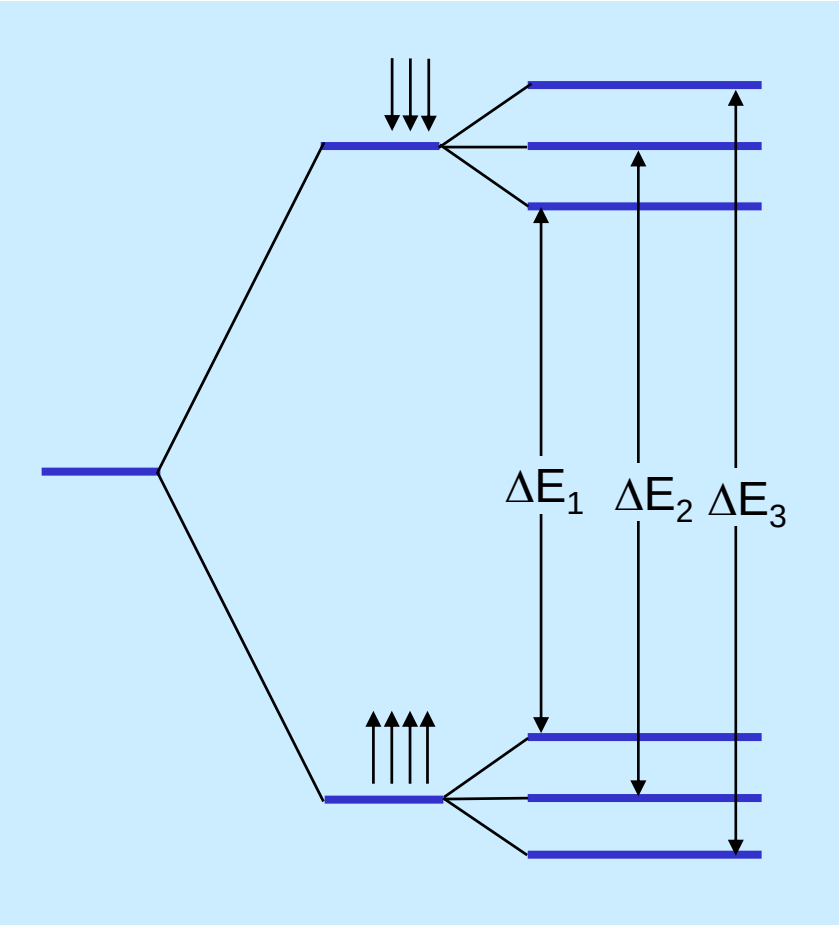



**Optimal Boltzmann distribution**

**Boltzmann distribution under "saturating" conditions**

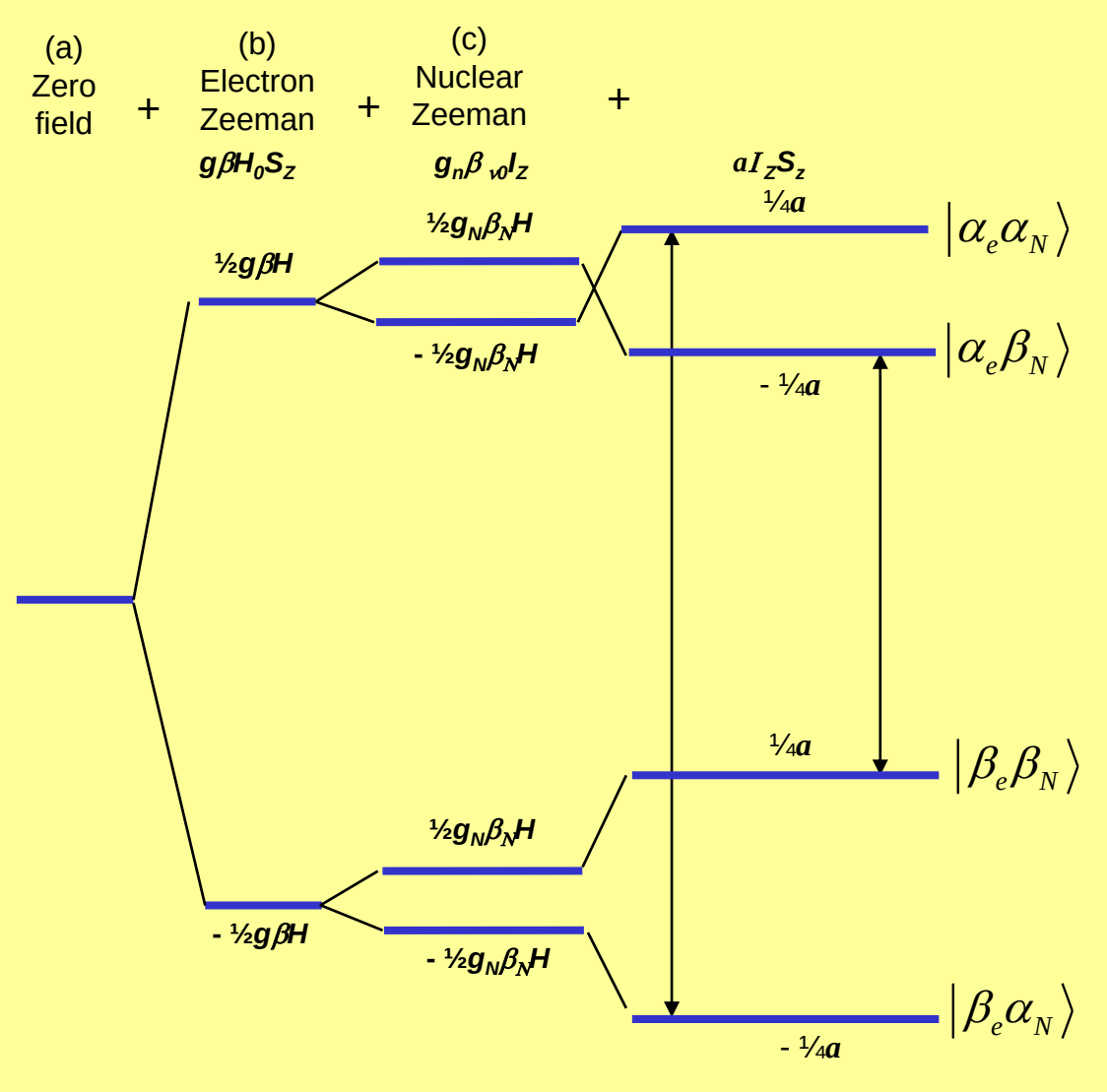


# Approximate Energy Levels for a $^{14}N$ Nitroxide Spin Label; S =½, I=1

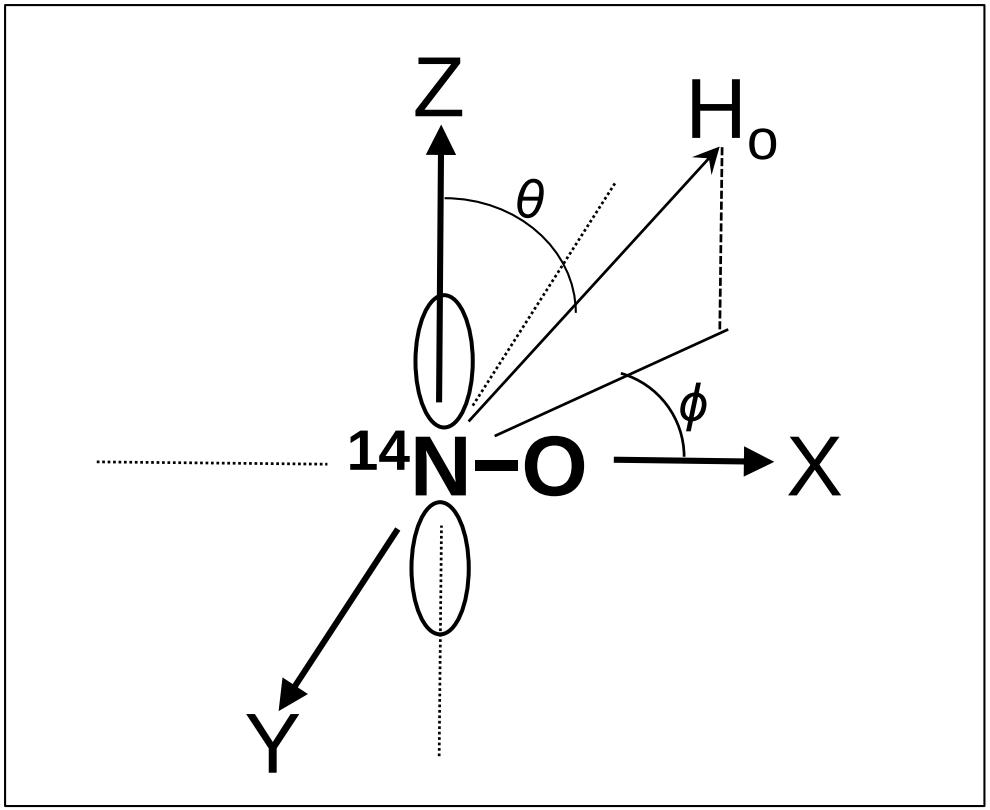



In general, EPR gives information on the # and nuclear spin state of nearby nuclei – coordination sphere of metal centers in in metalloproteins

# Energy level Diagram for a $^{15}N$ Nitroxide
## S=½; I=½



# Nitroxide Reference Frame




$g_{xx}$ = 2.0086
$g_{yy}$ = 2.0056
$g_{zz}$ = 2.0022
$A_{xx}$ = 7 G
$A_{yy}$ = 6 G
$A_{zz}$ = 34 G

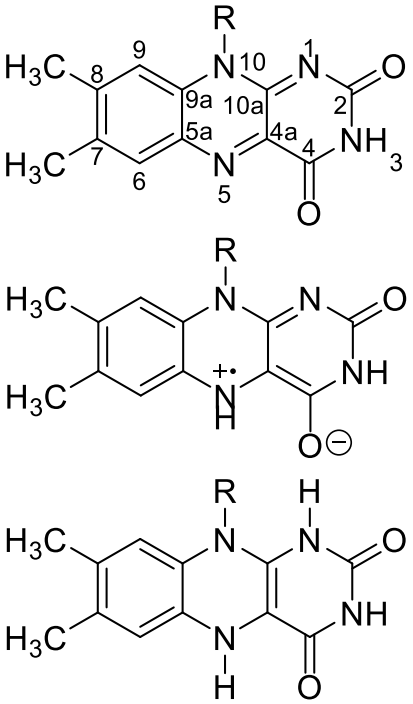
$$H_{res} = [h\nu/\beta_e g_{eff}(\theta,\phi)] + m_i A_{eff}(\theta,\phi)$$

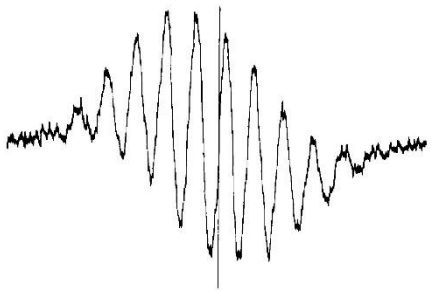
$$g_{eff} = g_{xx}\sin^2\theta\cos^2\phi + g_{yy}\sin^2\theta\sin^2\phi + g_{zz}\cos^2\theta$$

$$A_{eff} = [A_{xx}^2\sin^2\theta\cos^2\phi + A_{yy}^2\sin^2\theta\sin^2\phi + A_{zz}^2\cos^2\theta]^{1/2}$$

# Biological Relevant Simple Radicals

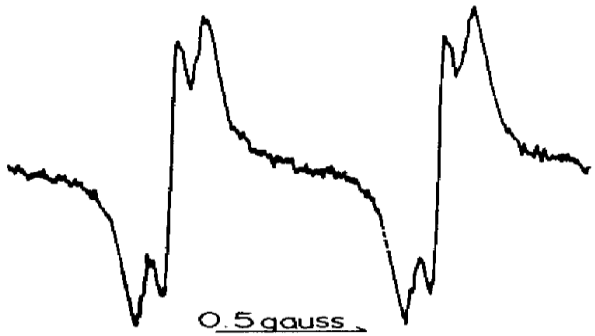
## FLAVINIC COENZIMES



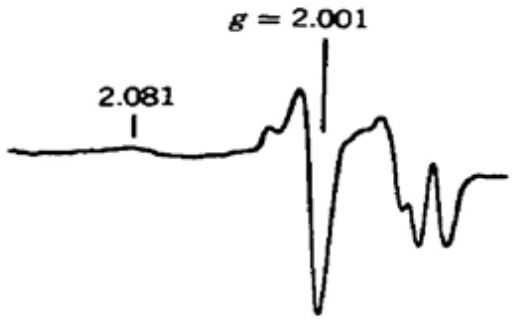
## MONODEHYDROASCORBATE

Redox equilibrium, continuous-flow



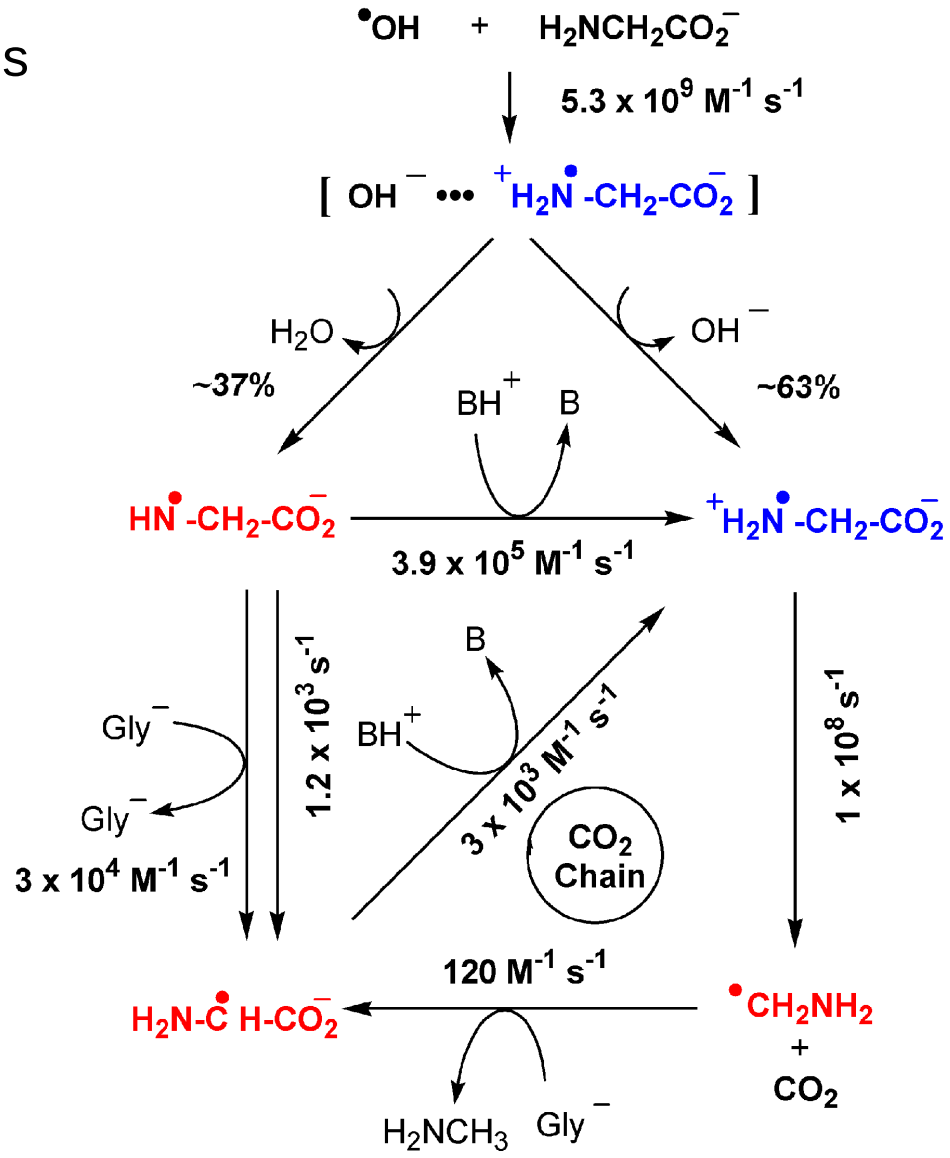

## SUPEROXIDE ANION

Stop-flow, rapid-freeze,77°K



# Kinetic Determinations

OH-oxidation of glycine anions




***Scheme*** from

Bonifacic, Stefanic, Hug, Armstrong, Asmus

*J. Am. Chem. Soc*. **1998**, *120*, 9930-9940

# Summary of Features Underlying ESR of Free Radicals (1)

1. An electron is a spinning unit of negative charge with a magnetic dipole.
2. Electrons paired in atomic or molecular orbitals have their intrinsic magnetism canceled out; hence, most organic molecules are diamagnetic.
3. Free radicals are paramagnetic because they have a net unpaired electronic magnetic moment.
4. An external magnetic field aligns free or unpaired electrons into one of two quantized states with respect to the orientation of the electronic magnetic moment to the field– parallel (a slightly lower energy state) and antiparallel.
5. A resonant high-frequency electromagnetic field (usually in the microwave range) excites spin flips between the two states.

# Summary of Features Underlying ESR of Free Radicals (2)

6. Net energy is absorbed from the radiating field because initially there are more electrons in the parallel-aligned state.
7. The essential statement of the ESR resonance condition: Resonating (microwave) field frequency ÷ applied magnetic field strength = $g \times$ (physical constants)
8. Major components of an ESR spectrometer: (1) scanning electromagnet, (2) microwave source and conductors, (3) sample cavity, and (4) sensitive detection and signal amplification.
9. Usual ESR spectra are the first derivatives of microwave power absorbed plotted vs. applied magnetic field strength.
10. Electron spin resonance spectral lines have shape, width, intensity and position (*g*-value)
11. Hyperfine spectral line splitting from the interaction of unpaired electrons with magnetic nuclei can determine the structure or positions of free radical components and is a powerful aid in free radical identification

POLITECNICO DI MILANO



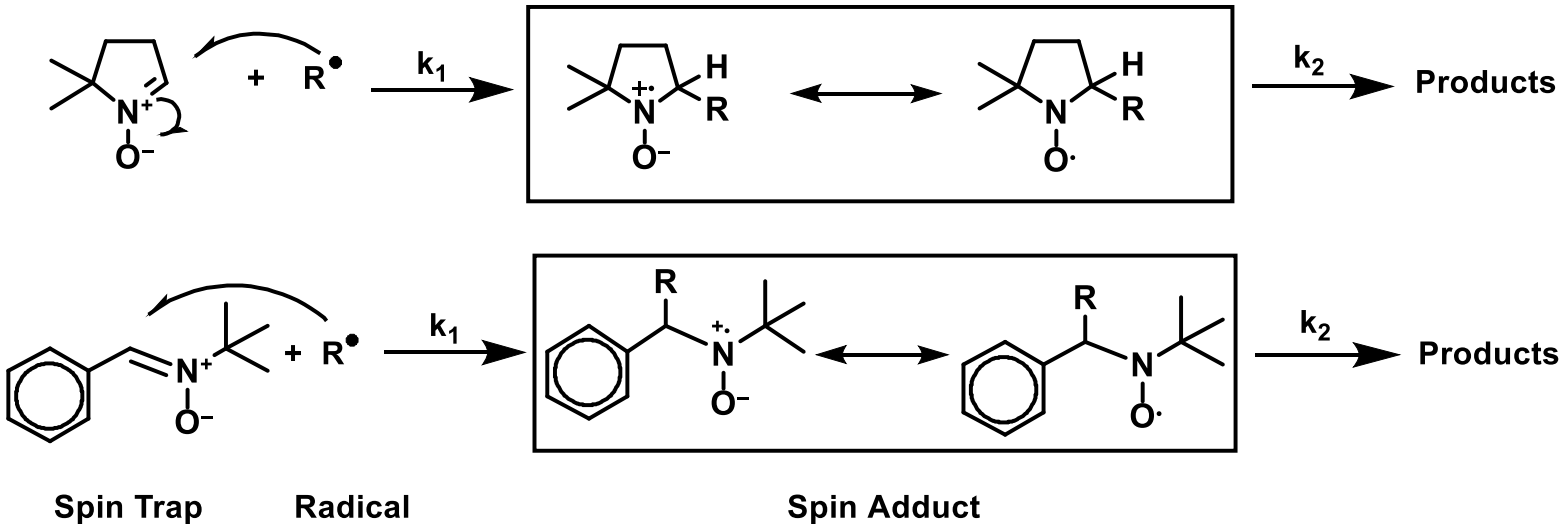


# ESR Detection of Radicals :
# C. Spin Trapping and Spin Labeling

Prof. Attilio Citterio
Dipartimento CMIC “Giulio Natta”

# Spin Trapping Technique

A diamagnetic spin trap (ESR silent) compound reacts with reactive short-lived free radicals to form a more persistent nitroxide or spin adduct. From the ESR spectrum of the spin adduct, the structure of the reactive free radical can be deduced indirectly.

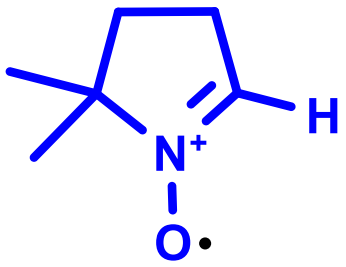



$R^{\bullet}$ = $HO^{\bullet}$, $O_2$ , $LOO^{\bullet}$, $LO^{\bullet}$, $RS^{\bullet}$, $C_3Cl^{\bullet}$, etc.

# Properties of a “Good” Spin Trap

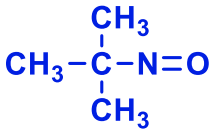
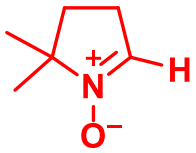
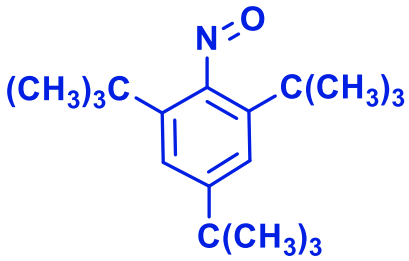
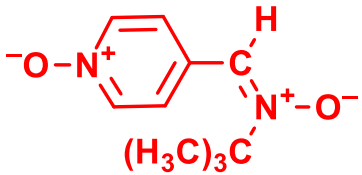
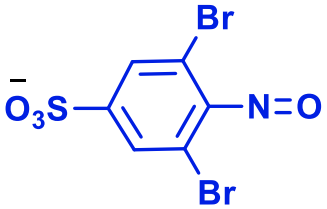
1. Stable (2 classes: nitroso R-N=O, and nitrones R-CH=N(O)R’)
2. Easy to purify
3. Spin adduct is relatively stable
4. Different spin adducts have distinctly different ESR spectra
5. not toxic

Most commonly cyclic nitrone used:




5,5-Dimethyl-1-pyrroline *N*-oxide (DMPO)

# A Selection of the Spin-Traps that have been Used in Biological System

| Name | Abbreviation | Structure |
|---|---|---|
| *tert*-Nirosobutane (nitroso-*tert*-butane) | tNB (NtB) |  |
| α-Phenyl-*tert*-butylnitrone | PBN | |
| 5,5-Dimethylpyrroline-*N*-oxide | DMPO |  |
| *tert*-Butylnitrosobenzene | BNB |  |
| α-(4-Pyridyl-1-oxide)-*N*-*tert*-butylnitrone | 4-POBN |  |
| 3,5-Dibromo-4-nitroso-benzenesulphonic acid | DBNBS |  |

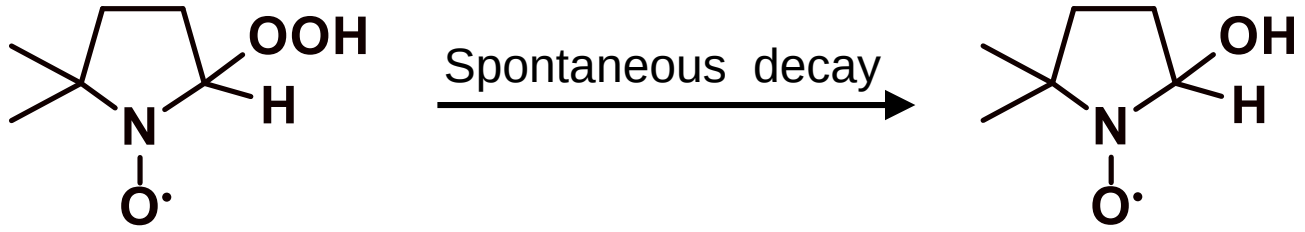
# DMPO Spin-trapping

## Advantages:

- Large data base

## Disadvantages:

- Impure liquid
- Needs additional purification by vacuum distillation or charcoal treatment
- Superoxide adduct is unstable




DMPO superoxide adduct

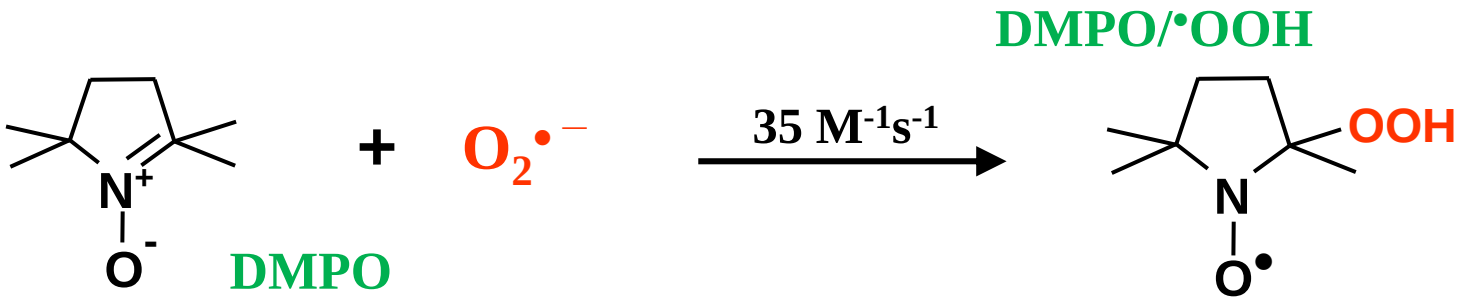
DMPO hydroxyl adduct

# Detection of $O_2^{\bullet -}$ with EPR spectroscopy

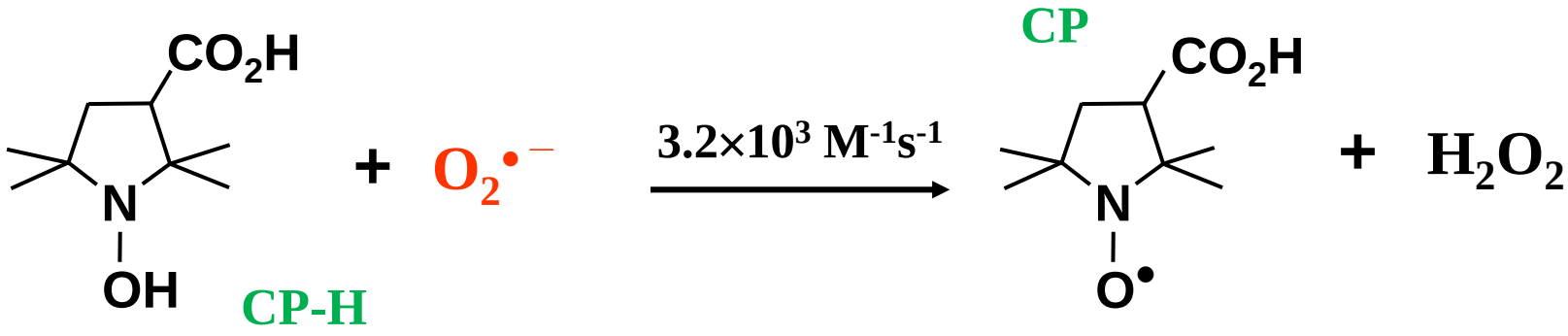
1. **Direct detection**

$$O_2 + 1\,e^- \longrightarrow O_2^{\bullet -} \xrightarrow{SOD} H_2O_2$$

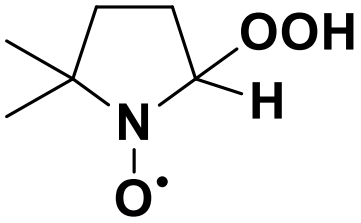
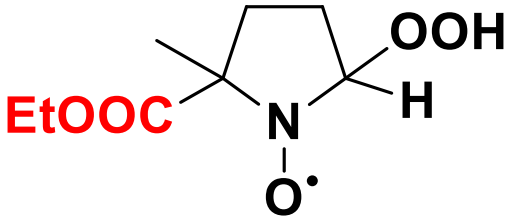
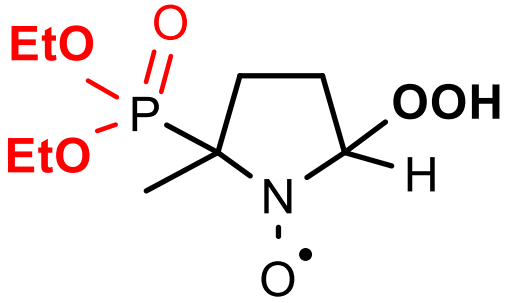
2. **Spin trapping (DMPO, EMPO, DEPMPO)**

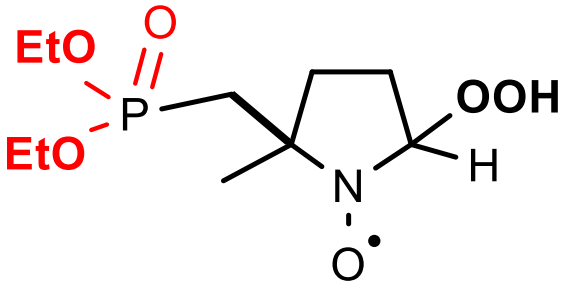
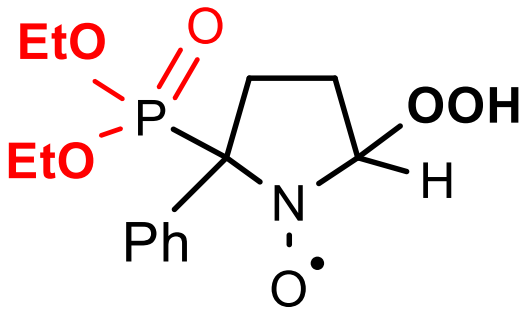
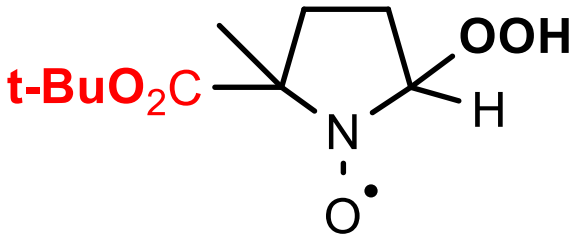



3. **Spin probes (cyclic hydroxylamines)**



# Relative Half-Lives of Superoxide Adducts

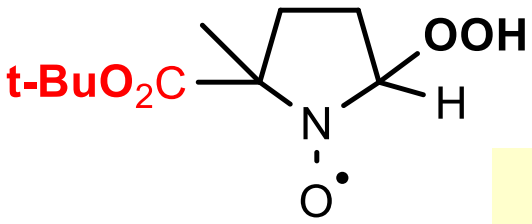
| | $t_{1/2}$ (min) |
|---|---|
|  | 1 |
|  | 8 |
|  | 14 |

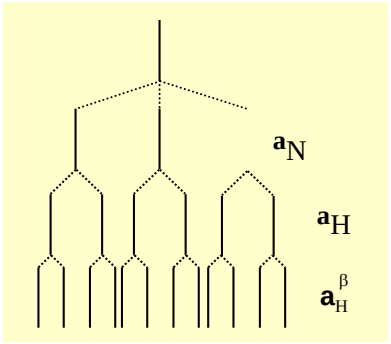
| | $t_{1/2}$ (min) |
|---|---|
|  | 3 |
|  | 13 |
|  | ? |

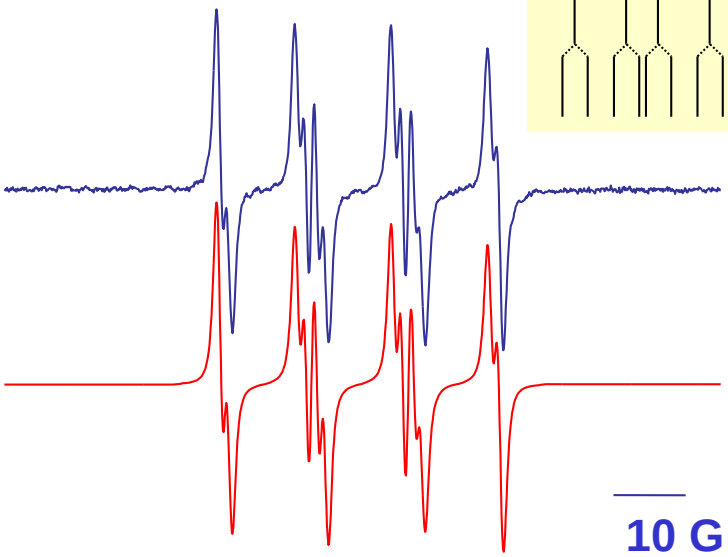
*FEBS Letters. 473(1):58-62, Free Rad. Biol.Med.. 28(3):403-408*

# Spin-trapping of Superoxide with BMPO Trap

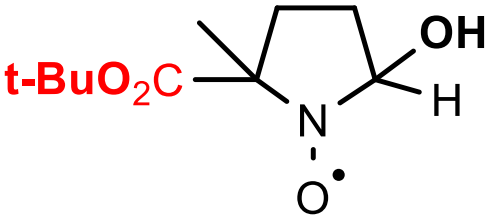
## BMPO-OOH adduct spectrum

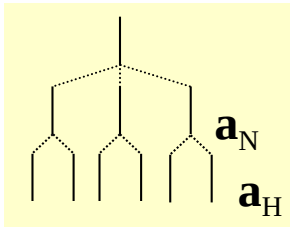


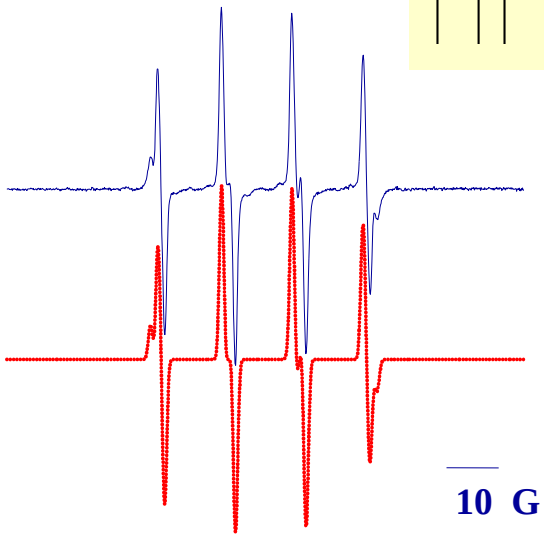



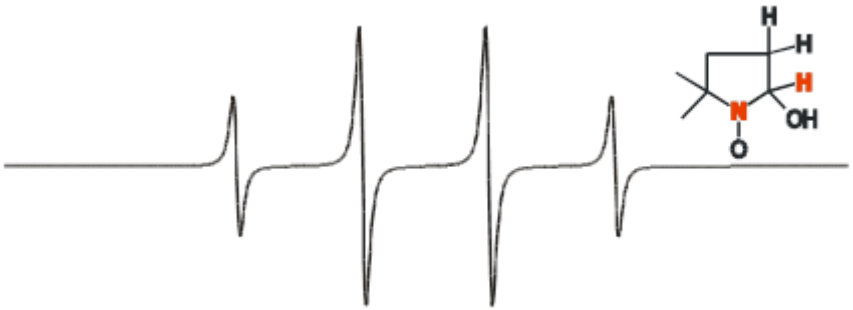

## BMPO-OH adduct spectrum

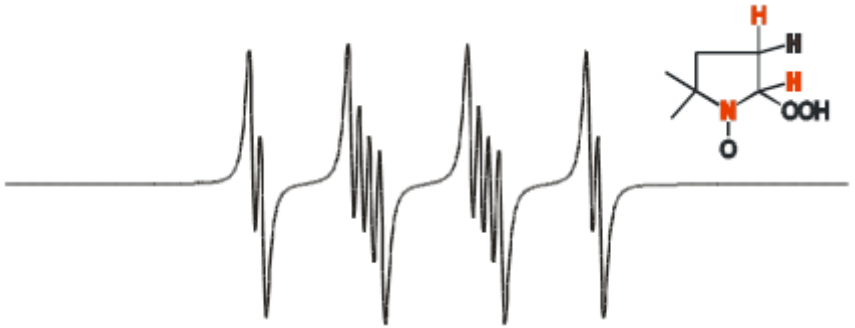


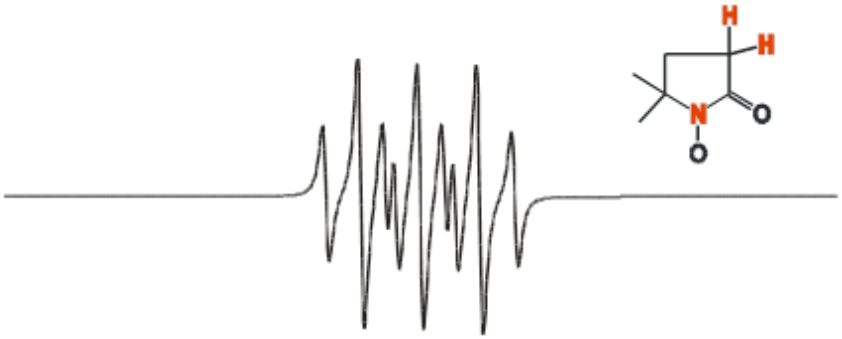



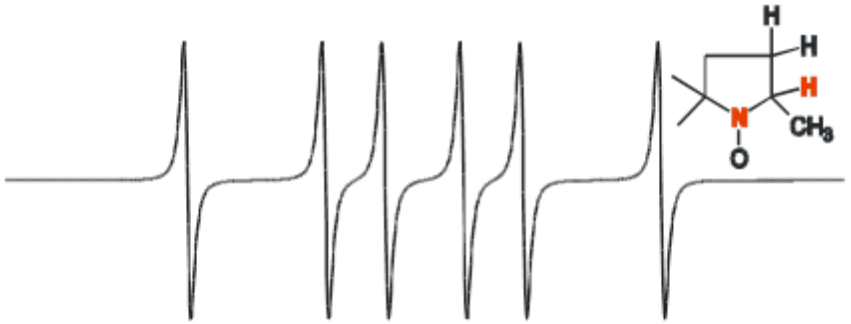



# EPR Spectra from DMPO Adducts

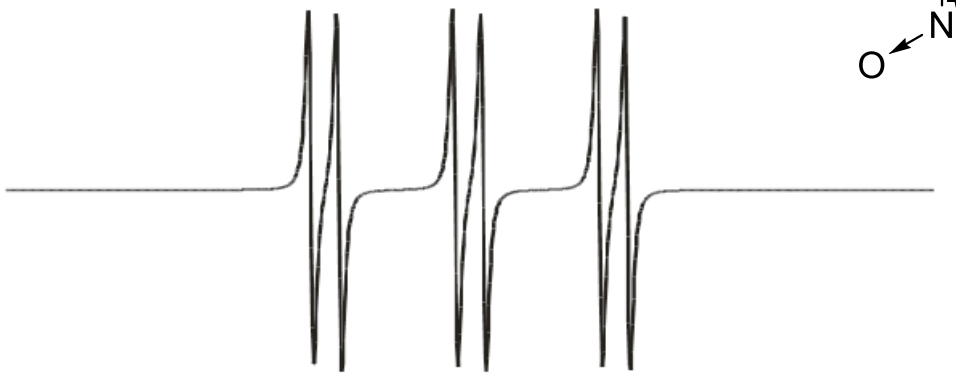


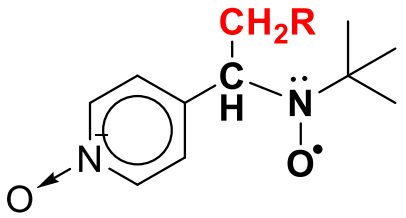



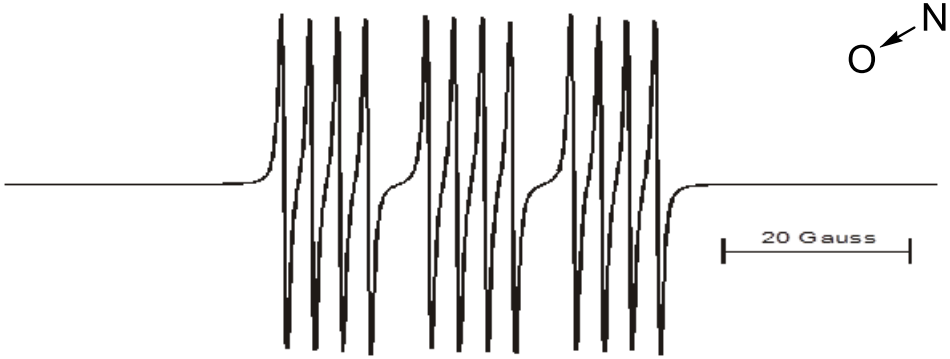



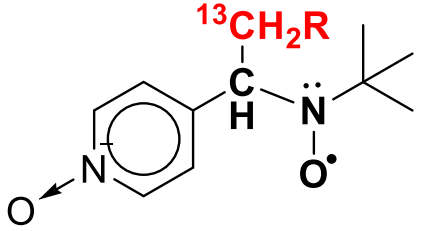



# EPR spectra from 4-POBN adducts

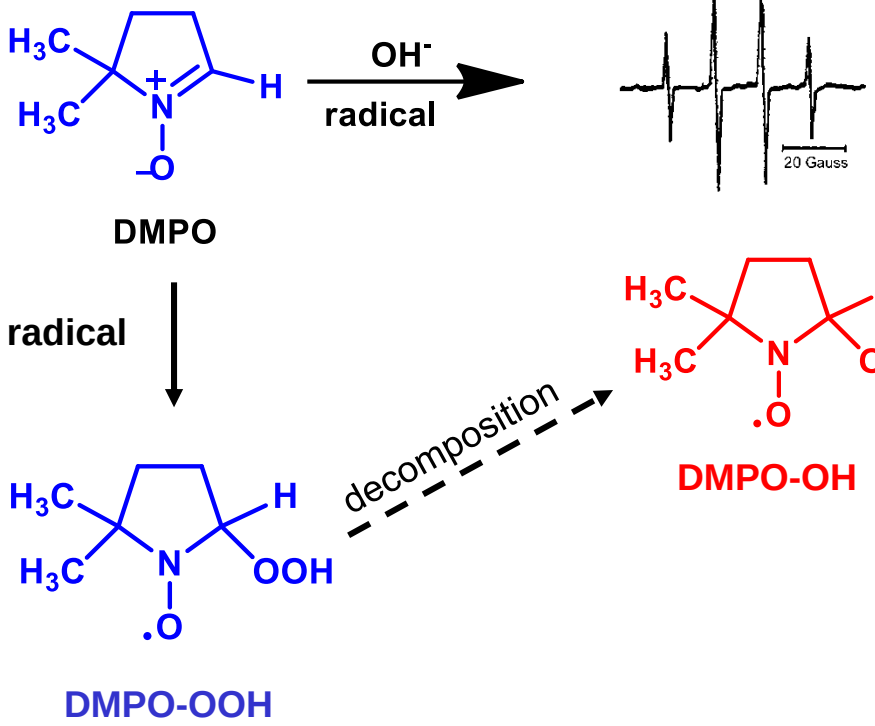




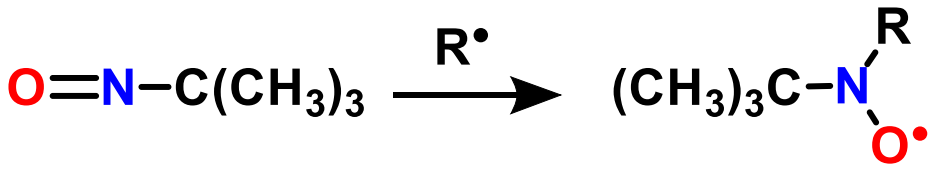



# Reactions of the Spin-Trap DMPO with Superoxide, Hydroxyl and Ethanol Radicals

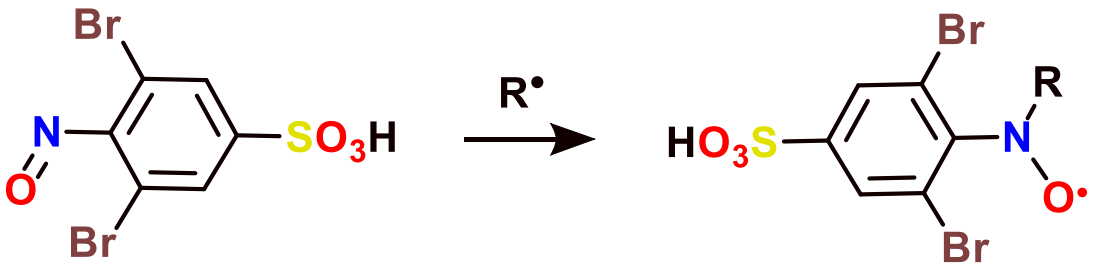


# Nitroso Spin Traps

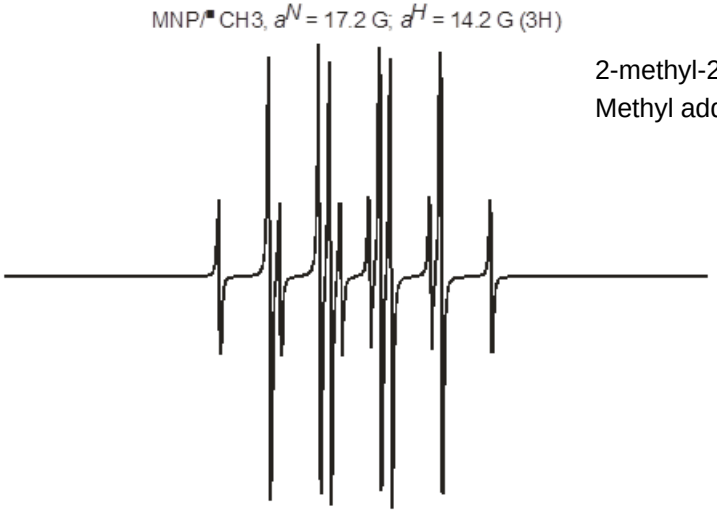
- Free radical adds to the nitrogen atom of a C-nitroso compound
- 2-methyl-2-nitrosopropane, MNP



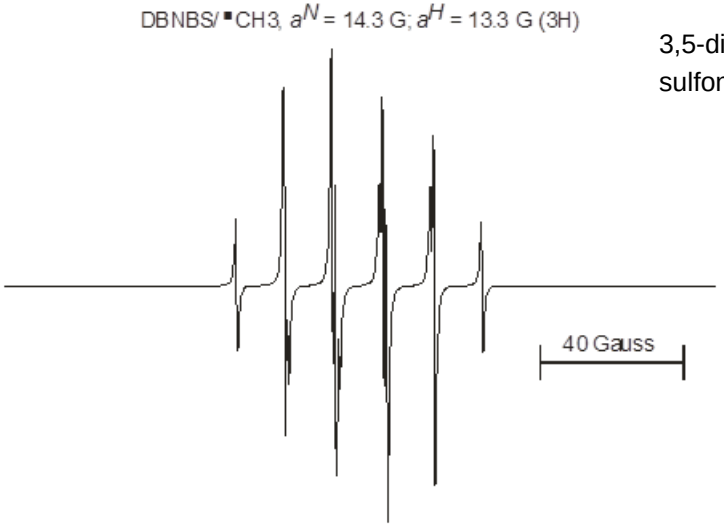

- 3,5-dibromo-4-nitrosobenzene sulfonate, DBNBS



# EPR Spectra from Methyl Radical Adducts of Nitroso Traps



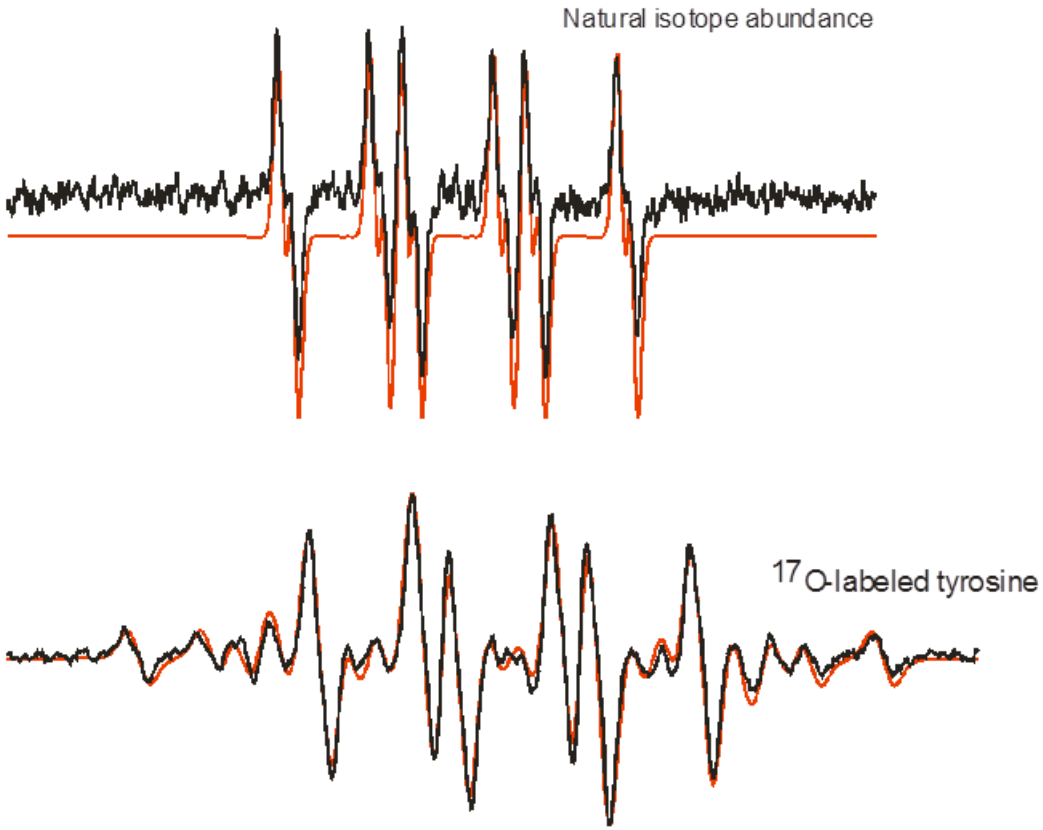

2-methyl-2-nitrosopropane
Methyl adduct

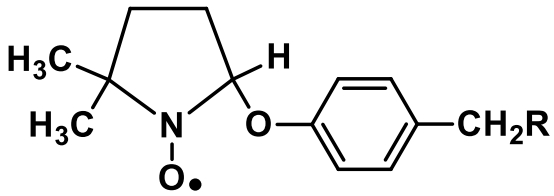



3,5-dibromo-4-nitrosobenzene
sulfonate Methyl adduct

# DMPO-trapping the Tyrosyl Radical

- Oxidize tyrosine with HRP/$H_2O_2$

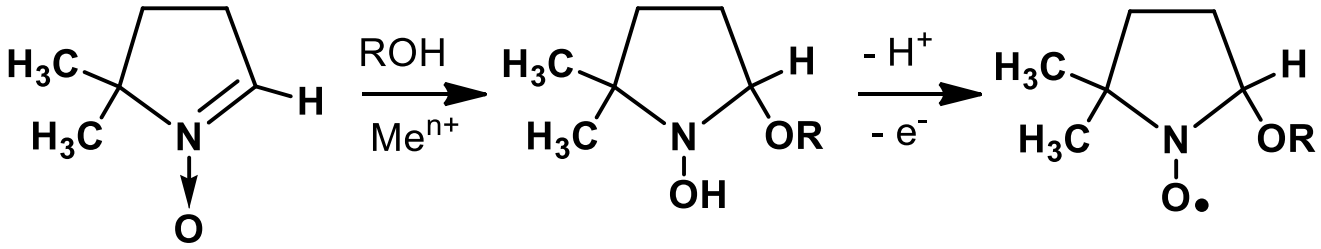



Gunther, M.R., et al., *Biochem. J*. **1998**, *330*, 1293-1299.

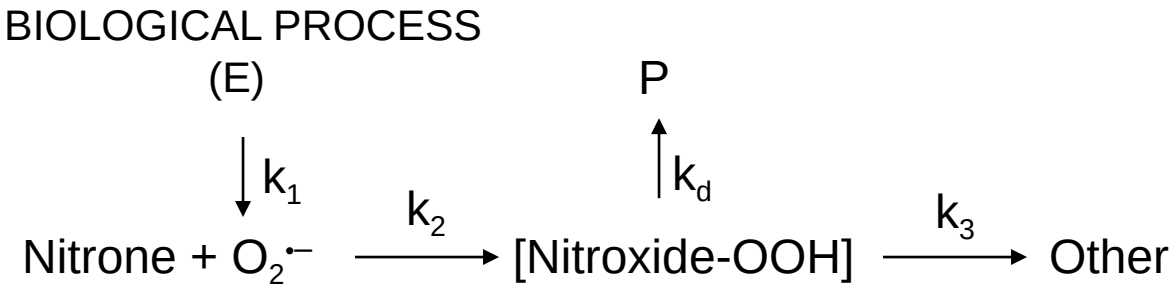
# Why not Spin Trap?

- Nitrone spin traps, especially DMPO
  - Adducts can interconvert, i.e., DMPO/·OOH decays to form DMPO/·OH
  - Subject to ***rare*** nucleophilic addition across their double bonds
  - Yields an EPR silent hydroxylamine which can be facilely oxidized up to the nitroxide




- Nitroso spin traps MNP and DBNBS
  - Often acutely toxic so can't use *in vivo*
  - The C-nitroso group critical to their function is highly reactive
  - Tend to directly add across unsaturated systems giving EPR-silent hydroxylamines that are readily oxidized to the corresponding nitroxides

# Superoxide Spin Trapping: Kinetic Analysis




$$\frac{d(Nitroxide-OOH)}{dt} = k_2(Nitrone)(O_2^{\bullet -}) - [k_3(Nitroxide-OOH) + k_d(Nitroxide-OOH)^2]$$

Under steady-state concentrations of superoxide and saturating concentrations of the nitrone, then

$$\frac{d(O_2^{\bullet -})}{dt} = k_1(E) - k_2(Nitrone)(O_2^{\bullet -}) = 0 \qquad \text{thus,} \qquad k_2(Nitrone)(O_2^{\bullet -}) = k_1(E)$$

and, $$\frac{d(Nitroxide-OOH)}{dt} = k_1(E) - [k_3(Nitroxide-OOH) + k_d(Nitroxide-OOH)^2]$$

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- Zhang H et al. (2000) Detection of superoxide anion using an isotopically labeled nitrone spin trap: potential biological applications. FEBS Lett 473: 58-62
- Zhao et al. (2001) Synthesis and biochemical applications of a solid cyclic nitrone spin trap: a relatively superior spin trap for detecting superoxide anions and glutathiyl radicals. Free Radical Biol Med 31:599-606

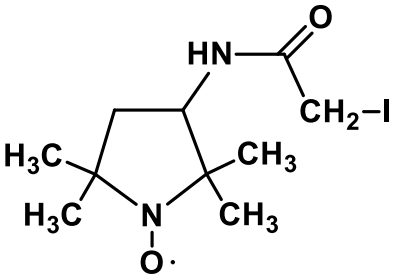

***Public EPR Software and Data Base***: http://epr.niehs.nih.gov/pest.html

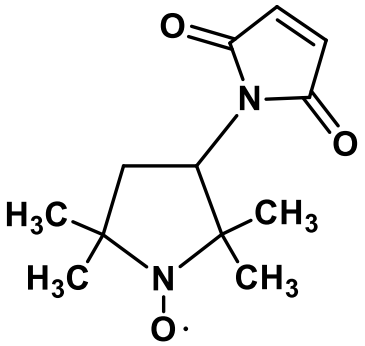
# Spin-labeling Technique

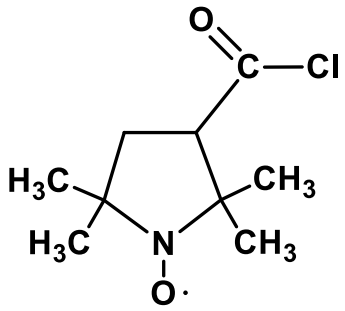
- Method using stable free radicals (NITROXIDES) to covalently mark diamagnetic biomolecules. The spin label work essentially as a probe of the surrounding.
- The efficiency of this function depend on the intrinsic properties of the nitroxide radical (Anisotropy of *g* and *A*, molecular geometry, Polarity)
- Owing to these features, EPR spectra of nitroxide are sensitive to structural peculiarity and to interactions of marked biomolecules in supra-molecular complexes. The spectral band changes allow to identify and measure these characteristics.
- The main limitations are related to the molecular weight ratio biomolecule/nitroxide: the system perturbation decrease with the increase of the molecular weight.

# Typical Nitroxide Radical Precursors for Spin Labeling

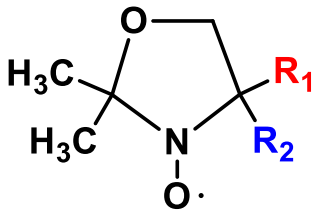
**Pirolidinil-N-oxil**
**Pirrolinil-**
**PROXIL**

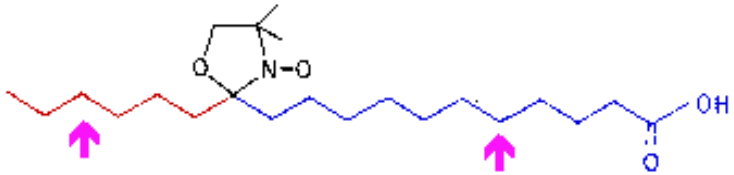




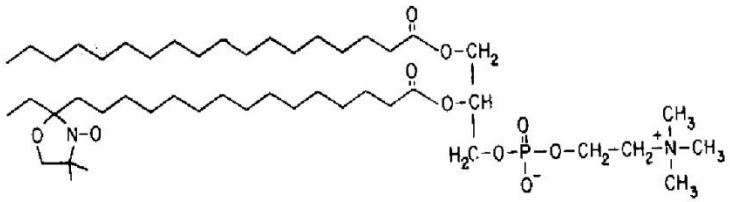


**Ossazolidinil-N-oxil**
**DOXIL**



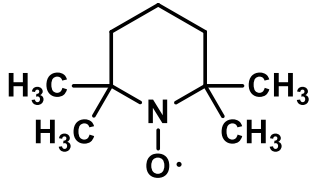


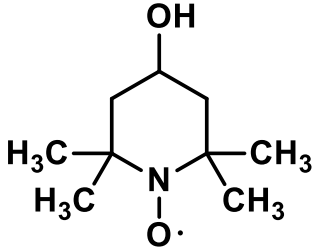
**(16-, 5-) 12-DSSA**

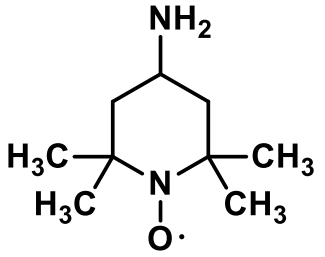


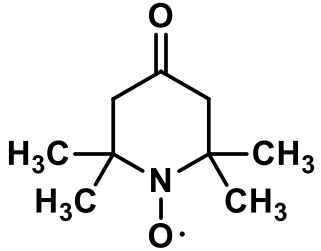
**16-DSPC**

**Tetrametilpiperidinil-N-oxil**
**TEMPO**





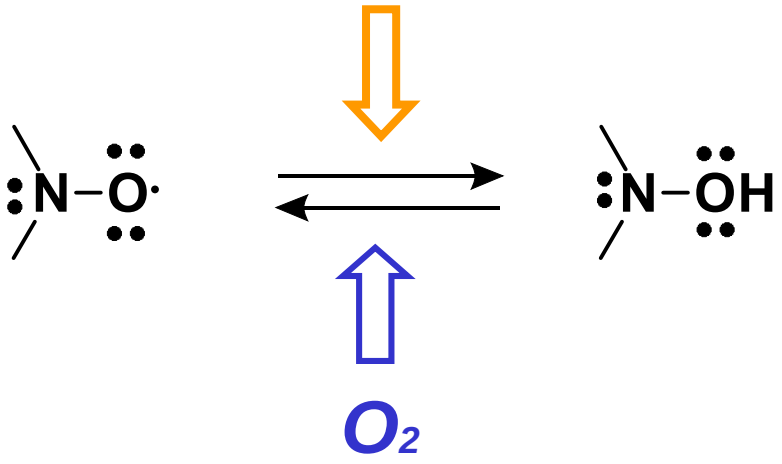




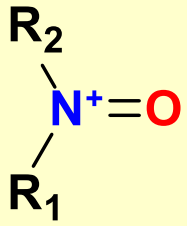
# Stability of Nitroxide Radical

***RIDUCTANTS:***

***Ascorbic Acid; Ditionite; Ti(III)/$H^+$; phenylhydrazine; thiols.***



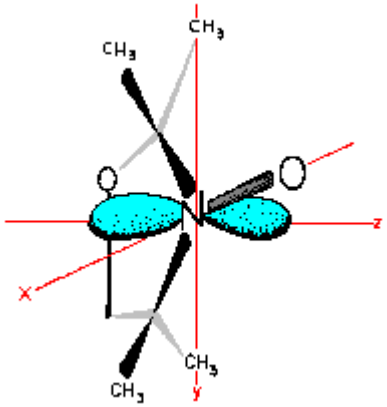

***Stable to: $LiAlH_4$ ; RCOCl; $SOCl_2$***

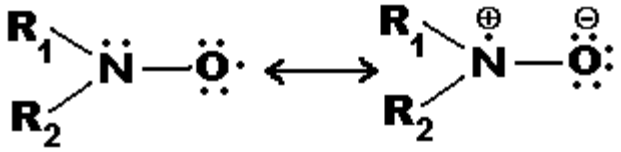
| | | |
|---|---|---|
| ***Over OXIDATION ($CrO_3$, $Ag_2O$)*** | ***oxoammonium salt,*** |  |



**L.J. Berliner, *Spin Labeling*, Academic Press]**

# Main Structural Features

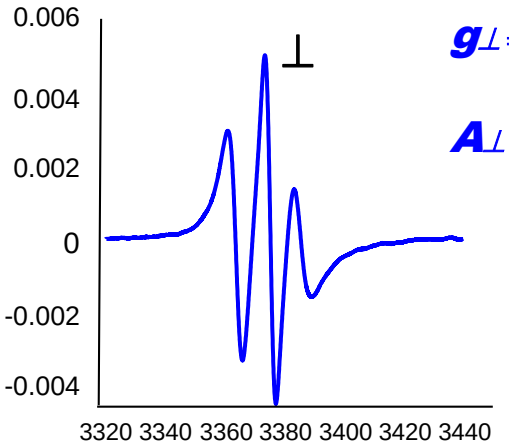



$I_N = 1;\ M_I = -1, 0, +1$

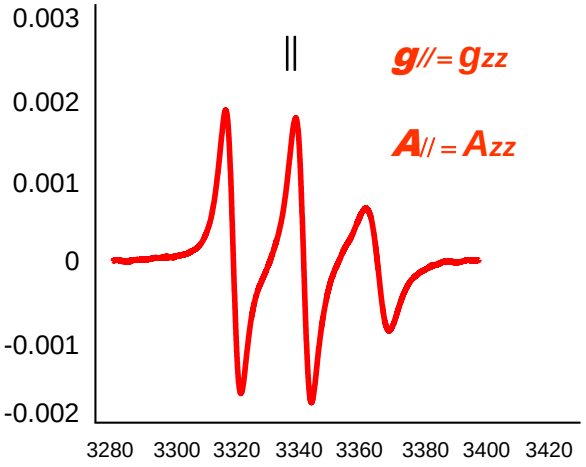
$$g = \begin{vmatrix} 2.0088 & 0 & 0 \\ 0 & 2.0061 & 0 \\ 0 & 0 & 2.0027 \end{vmatrix} \rightarrow \begin{vmatrix} 2.0075 & 0 \\ 0 & 2.0027 \end{vmatrix}$$

$$A = \begin{vmatrix} 6.3 & 0 & 0 \\ 0 & 5.8 & 0 \\ 0 & 0 & 33.6 \end{vmatrix} \rightarrow \begin{vmatrix} 6.05 & 0 \\ 0 & 33.6 \end{vmatrix}$$




$g_\perp = \frac{1}{2}(g_{xx} + g_{yy})$

$A_\perp = \frac{1}{2}(A_{xx} + A_{yy})$




$g_{//} = g_{zz}$

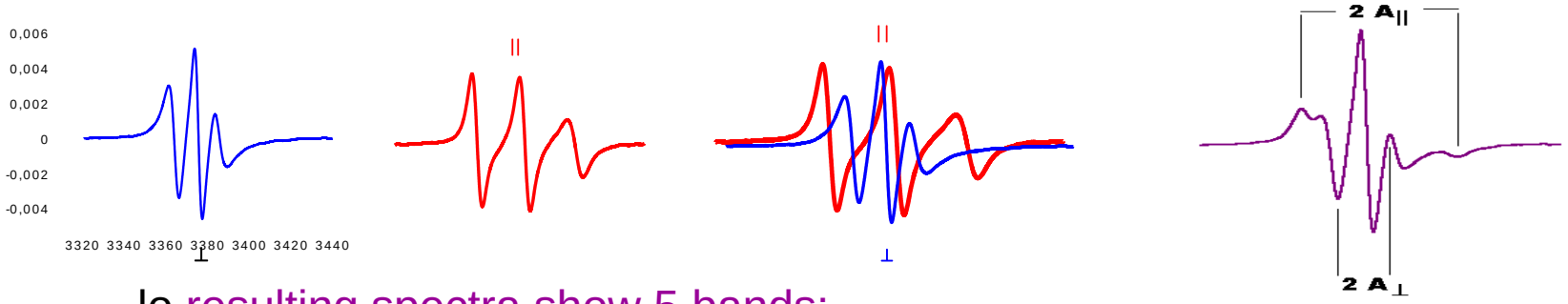
$A_{//} = A_{zz}$

# Sensitivity of Nitroxide to Biomolecule Mobility through *g* and *A* Anisotropy .

- Free molecular motion allows a *time averaging* of main diagonal elements of g and A tensors :
  - *g* = 1/3(*g*xx+*g*yy+gzz)
  - A = 1/3(Axx+Ayy+Azz)

the resulting EPR spectrum shows three equal bands:

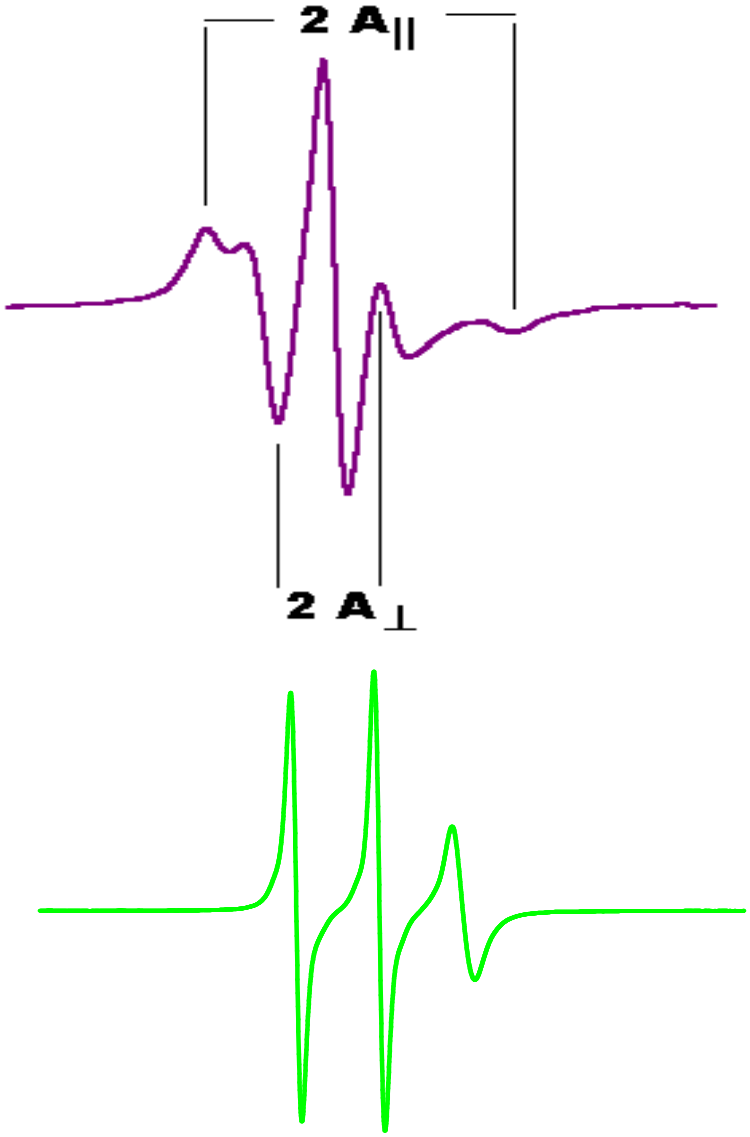
- Motion restriction prevent the *time averaging* and allows the simultaneous visibility of perpendicular and parallel spectra superimposed with the central bands coincident and external bands different:




lo resulting spectra show 5 bands:

Molecular motion modulate the anisotropy and the nitroxide EPR spectra will change depending on the dynamic state of the labeled biomolecule.

# Mobility Parameters




$$\tau_c > 10^{-7}\, s \qquad S = \frac{A_{\parallel} - A_{\perp}}{A_{xx} - \frac{A_{zz} + A_{yy}}{2}}$$

$$\tau_c < \nu^{-1}$$

***dynamic* Parameter**

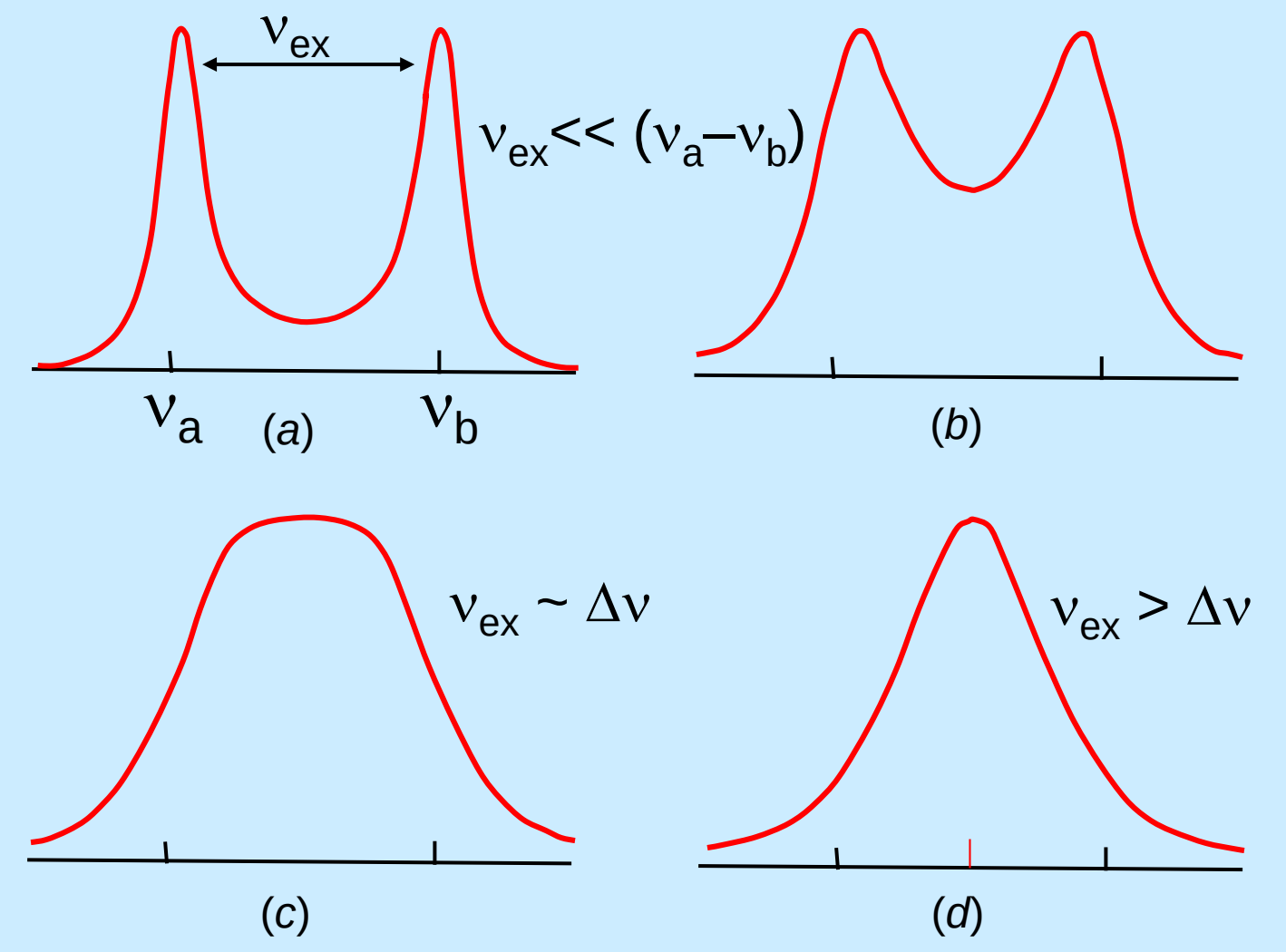
$$\tau_c \geq \nu^{-1} \qquad \tau_c = 0.652 \left[ \sqrt{\frac{h_0}{h_{+1}}} + \sqrt{\frac{h_0}{h_{-1}}} - 2 \right] \Delta H_0$$

***Axial symmetry; isotropic motion***

$$\tau_c = 4\pi\eta r^3 / 3kT$$

**[Debye]**

# Two-Site Exchange Model
## Effects of Motion on EPR Spectra



# Variation in the EPR Spectrum for a Single Nitroxide

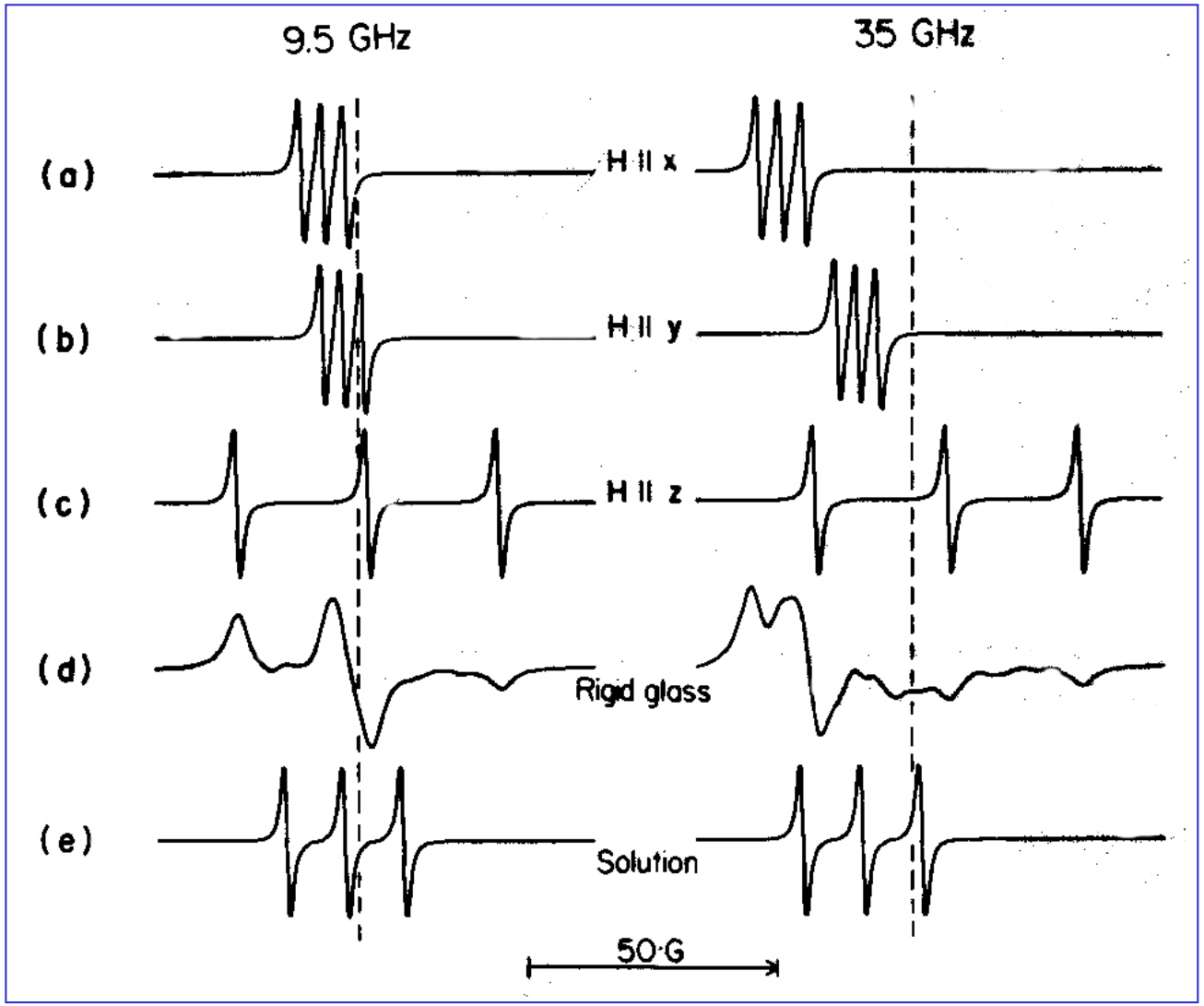
Single Crystal - x

Single Crystal - y

Single Crystal - z

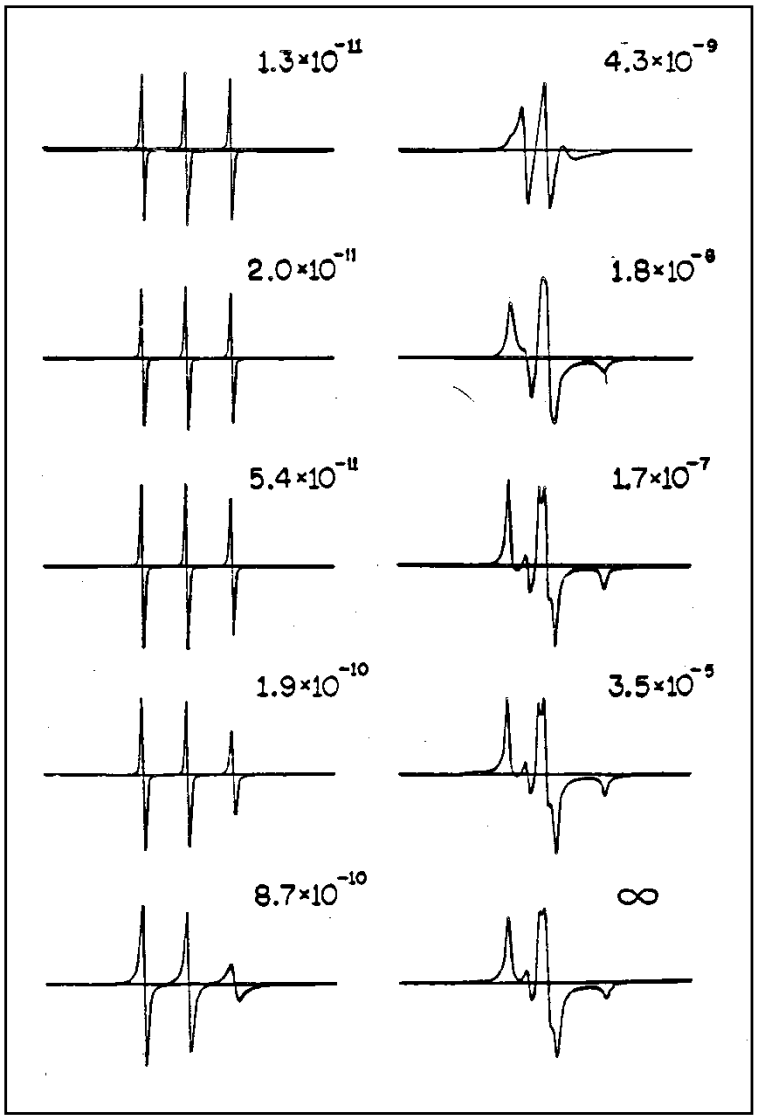
Polycrystalline

Crystal Dissolved in Water



# EPR Spectra as a Function of Rotational Correlation Time; $t_r = 1/6D_r$

$$\tau_r = 4\pi\eta r_h^3 / 3kT$$




Small Protein - EGF

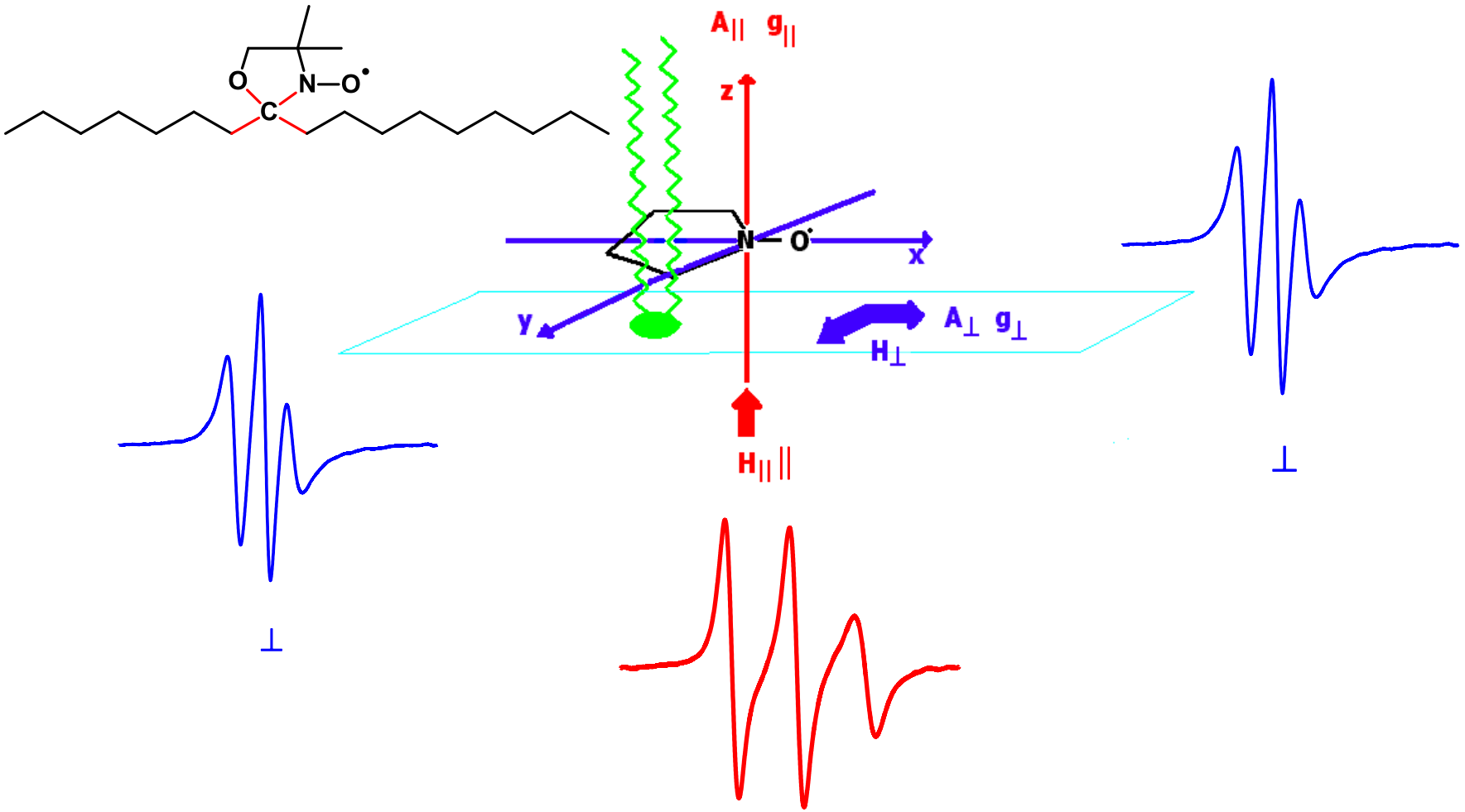
Nitroxide in $H_2O$

Large Protein – 1 MDa

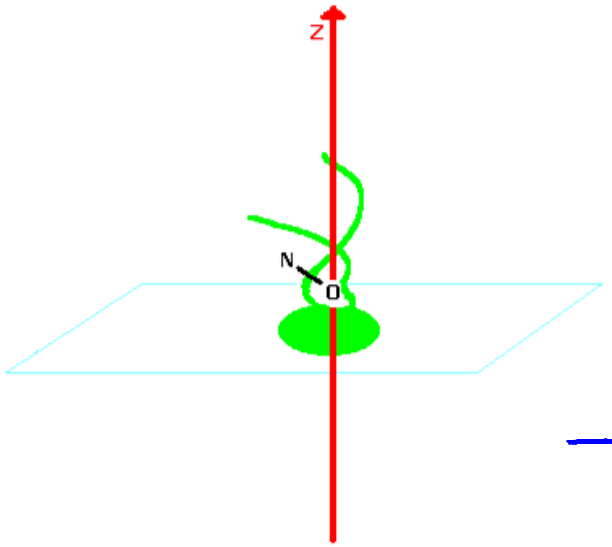
# Molecular Order

- When the motion is reduced (in the EPR time scale: $\tau_c << 1/\nu$) in a bilayer rigid(5-DSPC, S $\rightarrow$1) and oriented (planar membranes on a solid support, SPB), the nitroxide is able to see the geometric order of acylic chains of components phospholipids.
- Under molecular order conditions, the perpendicular and parallel spectrum are well different: an angular dependence (anisotropy) of EPR spectra are observed.
- However, la coexistence of $\perp$ and || spectra reveals the different orientation coexistence in phospholipidic bilayer, therefore disorder.
- The loss of perpendicular orientation of alkyl chains induces a loss of angular dependence with appearance of bands of || spectrum in the $\perp$ spectrum, and vice versa, until the total disappearance of an angular dependence of the EPR spectra.

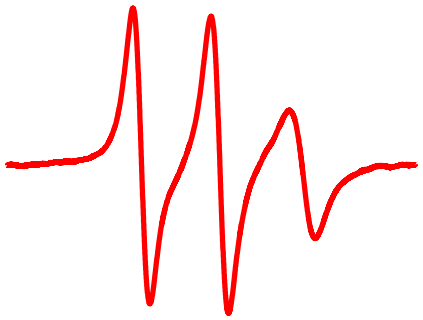
# Geometry of Phospholipid Spin Labels and EPR Spectral Anisotropy



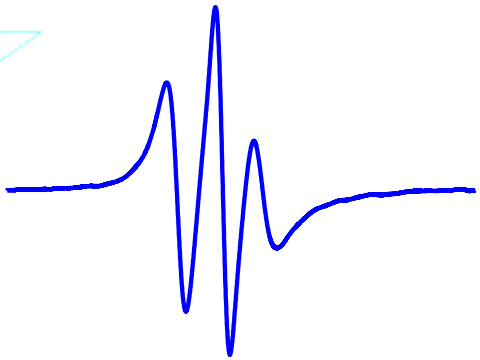
# Anisotropy Loss = Disorder




||

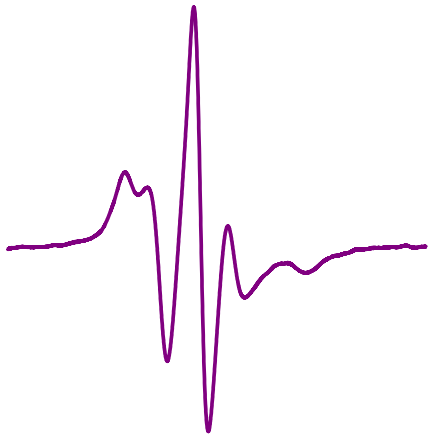


+



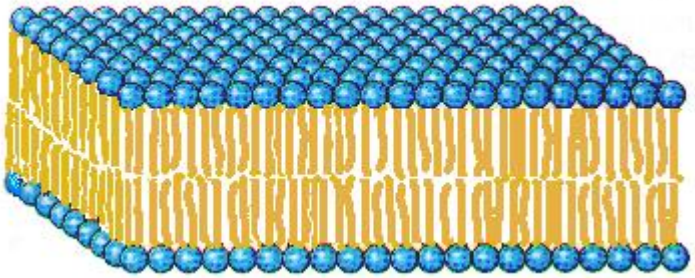
⊥

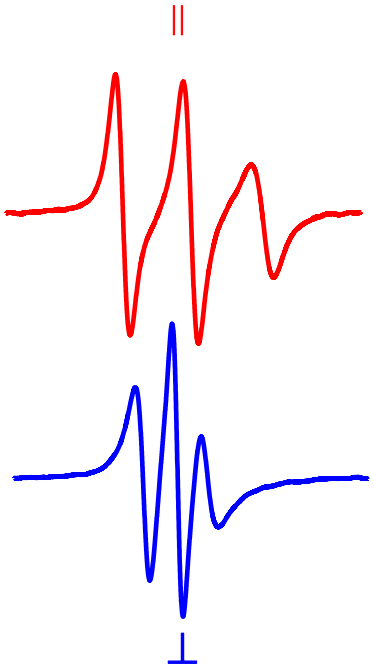
=

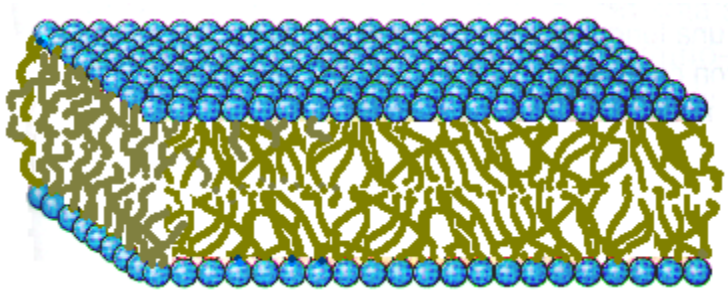


⊥ & ||

# SPB: order/disorder

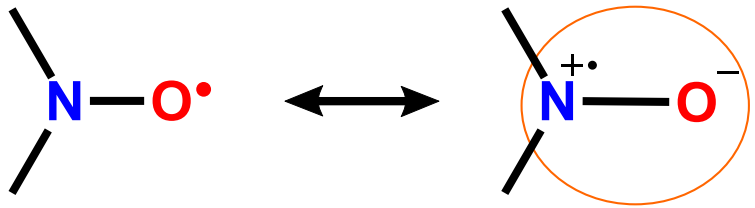




# Polarity and Hyperfine Coupling Constant

- One of the limit formula describing the electronic structure of nitroxide shows charge separation:




- This formula is stabilized in polar media and can be recognized in EPR spectra by an A value higher than in apolar media owing the higher electronic charge density on nitrogen atom:

$$A_0 = g_e \beta_e g_N \beta_N \frac{8\pi}{3} \psi^2_{(0)} \quad \text{(Hz)}$$

- Polarity measurement can be so carried out.

POLITECNICO DI MILANO





# ESR Detection of Radicals :
# D. Transition Metal

Prof. Attilio Citterio

Dipartimento CMIC “Giulio Natta”

# EPR of Transition Metal Proteins

- **Can be used to monitor metalloproteins which are paramagnetic – both Fe and Cu containing and not limited heme proteins**
- **Can distinguish between specific oxidation and ligation states and detect free radicals present on metalloproteins (spin traps)**

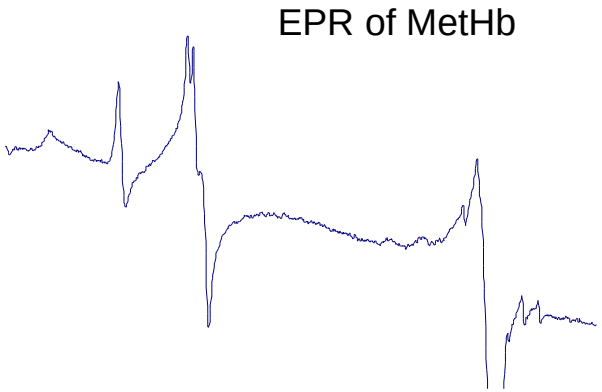
ferrous ($Fe^{2+}$),
ferryl ($Fe^{4+}$),
cuprous ($Cu^{+}$)

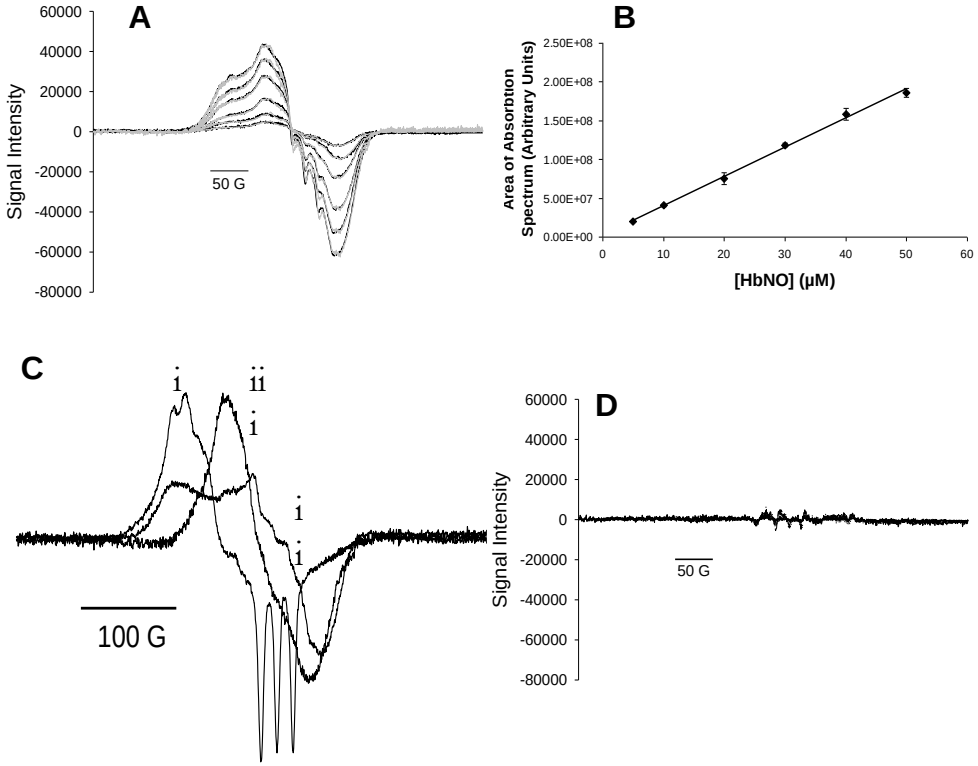
**EPR Silent**

ferrous-nitrosyl ($Fe^{2+}$)-NO,
ferryl-associated radicals
ferric ($Fe^{3+}$),
cupric ($Cu^{2+}$)

**Paramagnetic**
**Distinct EPR spectra**

# Reaction of NO with $MbO_2$ and Hb




$$HbO_2 + \bullet NO \xrightarrow{3.7x10^7\ M^{-1}s^{-1}} metHb + NO_3^-$$

$$Hb + \bullet NO \xrightarrow{2.6x10^7\ M^{-1}s^{-1}} HbNO$$

# Iron-sulfur Proteins: Escherichia coli NADH:Ubiquinone Oxidoreductase

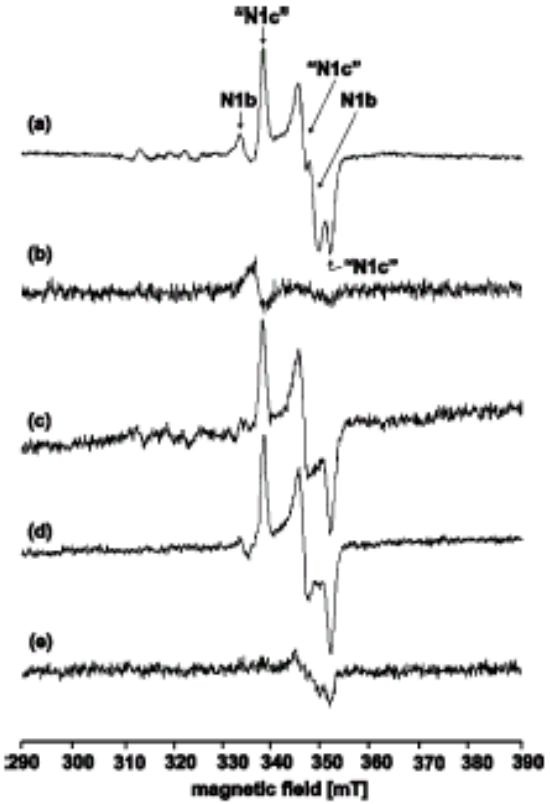



FIGURE 2: EPR (NADH-reduced minus air-oxidized) difference spectra of the cytoplasmic fraction from *E. coli* wild type (a) and from the NuoG mutant strains $Cys^{230}Ala$ (b), $Cys^{233}Ala$ (c), $Cys^{237}$-Ala (d), and $Cys^{265}Ala$ (e) at 40 K and 10 mW microwave power.

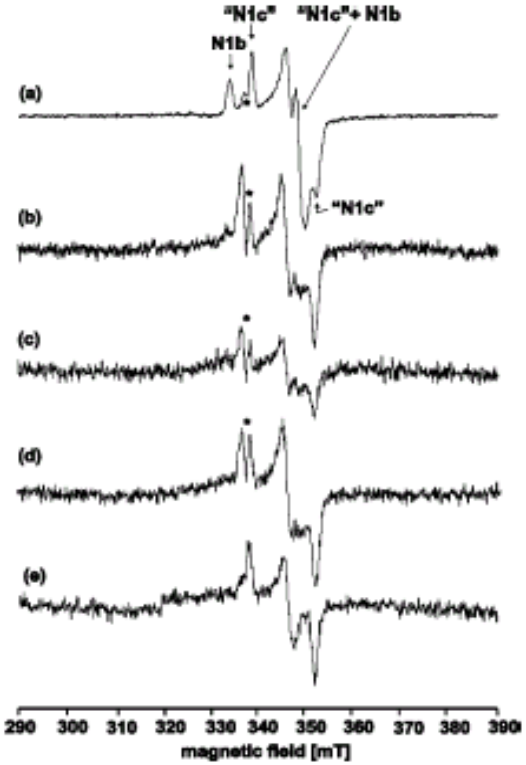


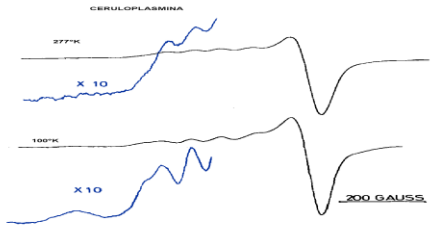

FIGURE 5: EPR spectra of the NADH dehydrogenase fragment purified from wild type (a) and from the NuoG mutants $Cys^{233}Ala$ (b), $Cys^{237}Ala$ (c), and $Cys^{265}Ala$ (d). The EPR spectrum of the NuoEF subcomplex isolated from the mutant $Cys^{237}Ala$ is shown in (e). Spectra were recorded at 40 K and 2 mW. The samples

The soluble NADH dehydrogenase fragment represents the electron input part of the complex and consists of the subunits NuoE, F, and G. **The FMN and four iron-sulfur clusters have been detected in this fragment by means of EPR spectroscopy.**

# Copper Proteins

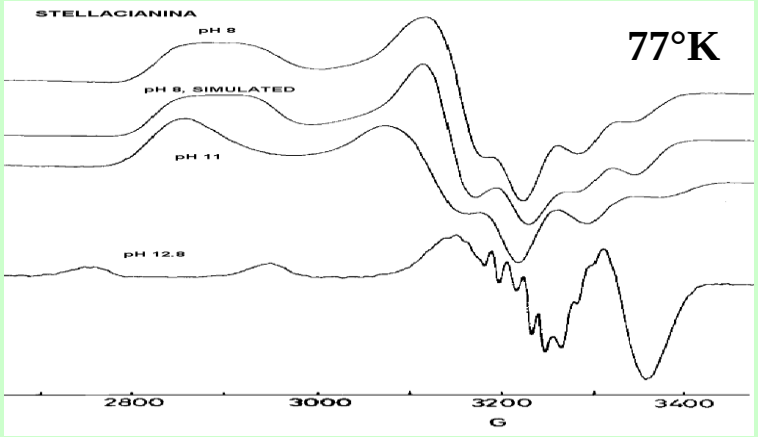
## CERULOPLASMINE

*Main Copper-protein of human sera;*
*redox factor of non ferrous substrates.*

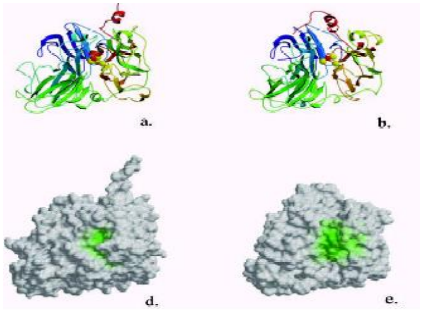


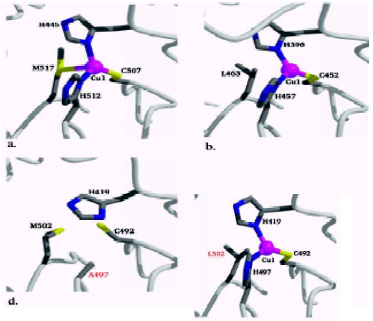

## STELLACIANINA

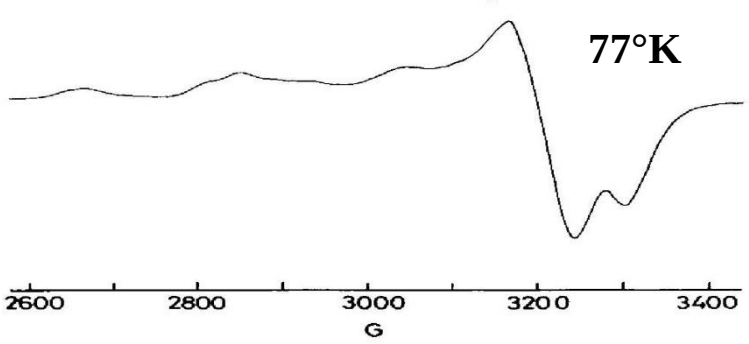
**The binding of small exogenous ligands to mutants of the blue copper protein stellacyanin from *Pseudomonas aeruginosa,* altered in the axial position, Met121X (X ) Gly, Ala, Val, Leu, or Asp), has been studied with optical and electron paramagnetic resonance (EPR) spectroscopy. The results show that small molecules can enter the pocket left by the side chain of Met121.**



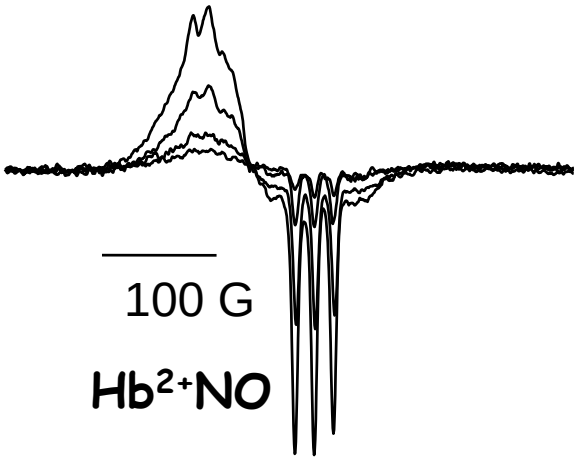

## LACCASES (Fungal and Bacterial Protein)



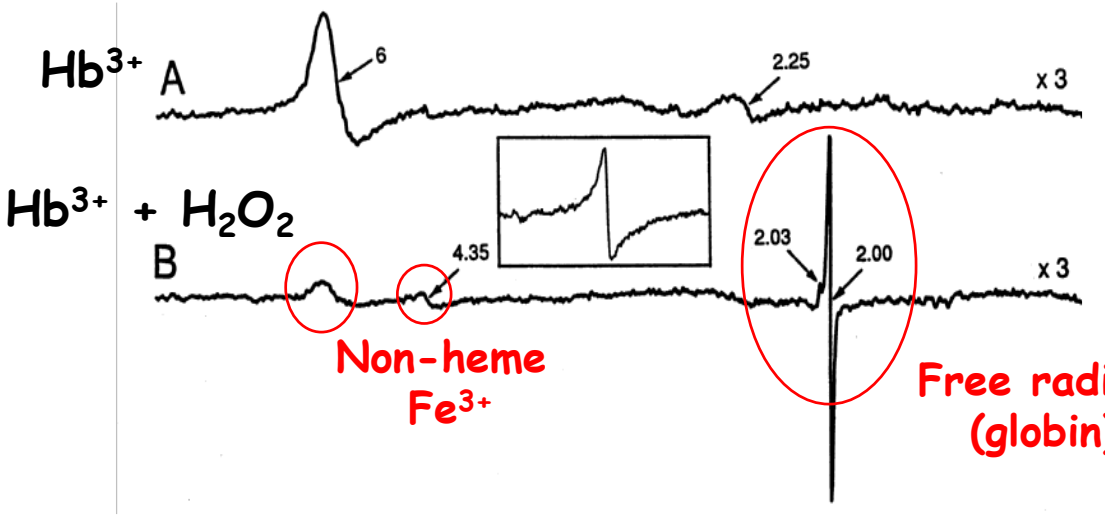



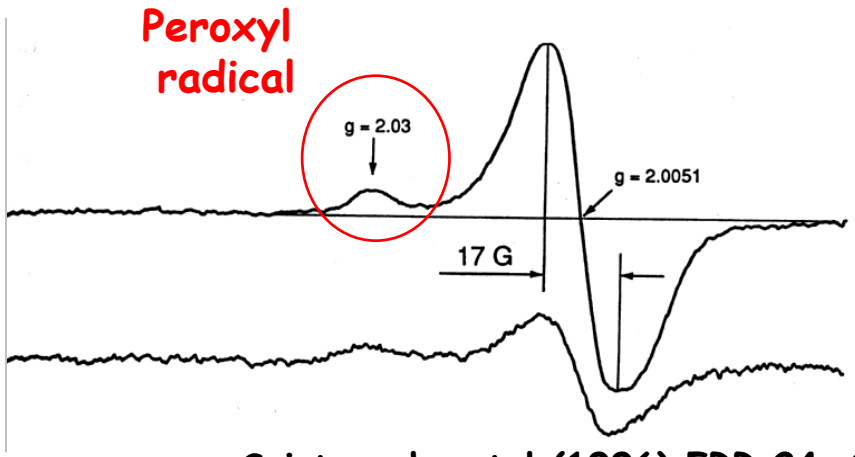



# Example: Identification of Redox and Ligation States



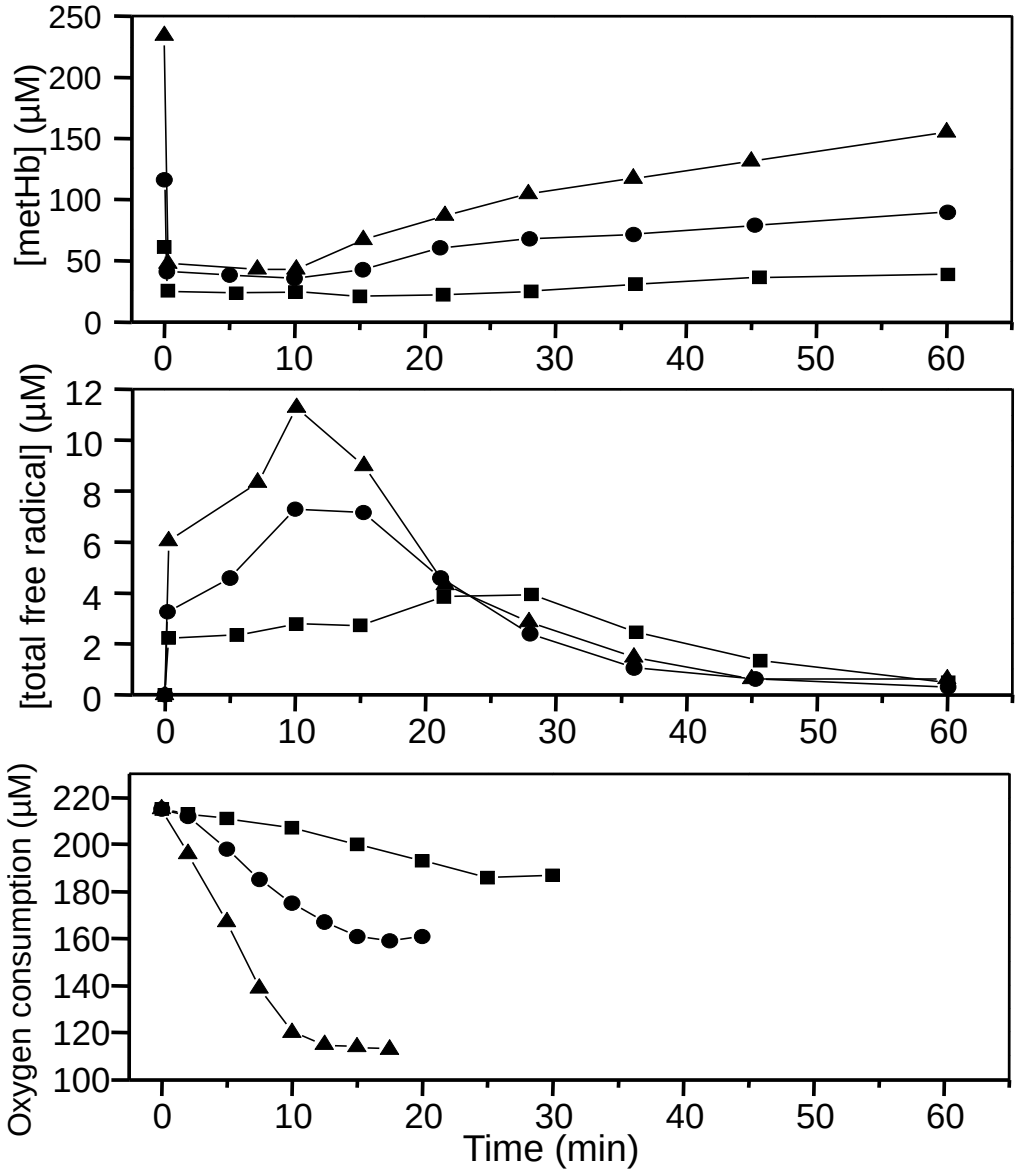

Spencer et al, (2000) JBC 275, 36562




Svistunenko etal (1996) FRR 24, 269

# Determining Mechanisms using Combination of UV/Vis, EPR and Polarographic Methods




$Hb^{3+} + H_2O_2$

$O_2$ consumption parallels peroxyl radical formation

When $O_2$ consumption ceases, peroxyl radical begins to decay and metHb concentrations start increasing