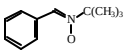
Spin trapping

- **Spin trapping** is a technique employed in the study of free radicals that are either unstable or terminate rapidly at ambient temperatures.
- Spin trapping involves the addition of a diamagnetic radical scavenger (the spin trap) to a reaction mixture containing a radical or radicals of interest.
- A reactive free radical adds to the scavenger, forming a long-lived paramagnetic adduct.
- This product can then be studied by EPR spectroscopy and the identity of the species adducted can be inferred from EPR spectral characteristics
  - such as an observed *g* value or a hyperfine-coupling pattern.

Spin traps

- The compound of interest has a very small lifetime & is therefore invisible in the ESR spectrometer.
- Trap it with a diamagnetic reactant to form a new more stable compound *but* still containing an unpaired electron.
- 2 major classes of traps: **nitrones** and **nitroso** compounds



N-tert-Butyl Phenyl-nitr

5,5-Dimethyl-1-pyrroline N-ox

Selection of the Spin Trap

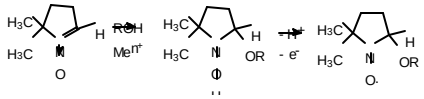
- Stable and easy to purify
- Radical adduct is persistent
- Radical adducts present distinctive EPR spectra
- EPR spectra is simple

A selection of the spin-traps that have been used in biological system

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

Nitrone spin traps

- 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



Advantage of the Nitrones

- React with a variety of different free radicals to form nitroxide adducts
  - RC·, RO·, RS·, in some cases RN·
- Adducts are often quite stable
- Not terribly toxic so amenable to *in vivo/ex vivo* spin trapping

Commonly Used Nitrones

- **DMPO**
  5,5-Dimethyl-1-pyrroline-N-oxide
  2,2-Dimethyl-3,4-dihydro-2*H*-pyrrole 1-oxide

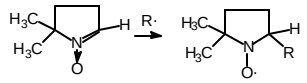
- **DEPMPO**
  5-(Diethoxyphosphoryl)-5-methyl-1-pyrroline-N-oxide
  2-Diethylphosphono-2-methyl-3,4-dihydro-2*H*-pyrrole 1-oxide
  (2-Methyl-3,4-dihydro-1-oxide-2*H*-pyrrol-2-yl) diethylphosphonate

- **EMPO**
  2-Ethoxycarbonyl-2-methyl-3,4-dihydro-2*H*-pyrrole-1-oxide
  5-ethoxycarbonyl-5-methyl-1-pyrroline N-oxide

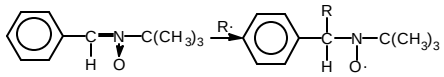
- **BMPO**
  5-*Tert*-butoxycarbonyl-5-methyl-1-pyrroline N-oxide

Nitrone spin traps

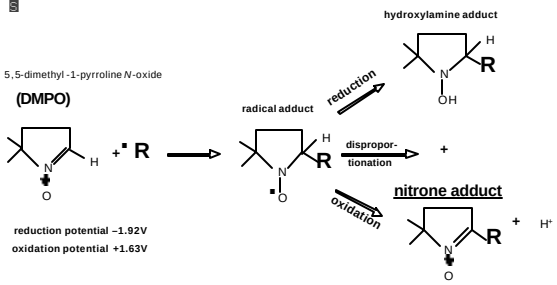
- DMPO, 5,5-dimethylpyrroline *N*-oxide



- PBN/4-POBN, phenyl-N-*t*-butylnitrone

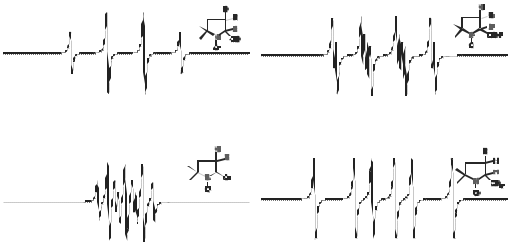


Trapping of Radicals with DMPO

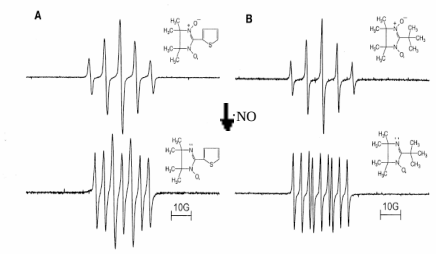


The specificity of the reaction of nitrone spin traps with free radicals has already made spin trapping with ESR detection the most universal, specific tool for the detection of free radicals in biological systems.

EPR spectra from DMPO adducts

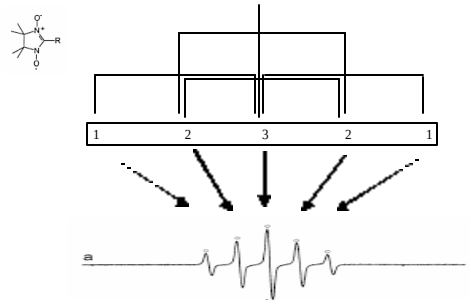


Nitrone detection



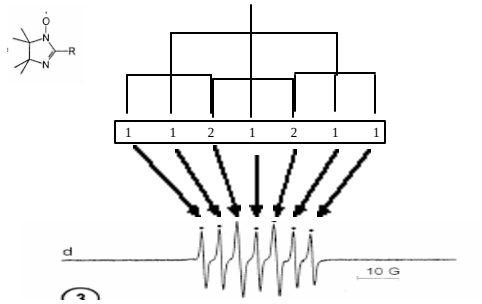
Origin of EPR spectrum

(NNO with two equivalent N centers)



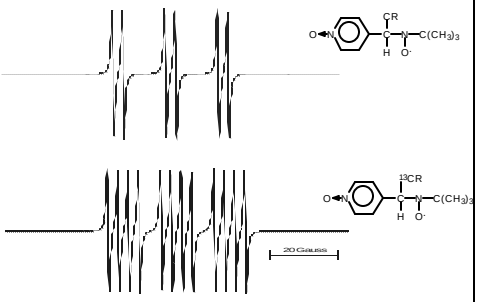
Origin of EPR spectrum

(INO with two nonequivalent N centers)



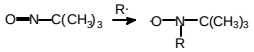
EPR spectra from 4-POBN adducts

phenyl-N-*t*-butylnitrone

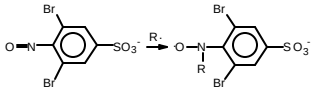


Nitroso Spin Traps

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

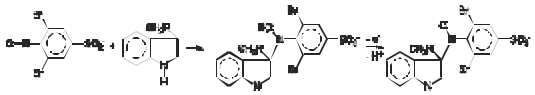


- 3,5-dibromo-4-nitrosobenzene sulfonate, DBNBS



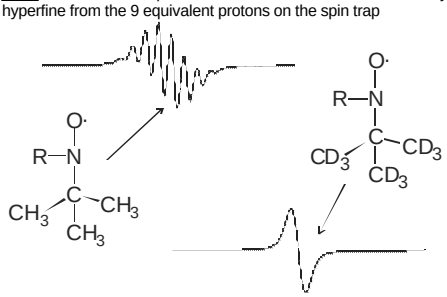
Nitroso spin traps

- 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



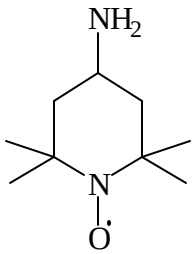
Spin trap-derived hyperfine from MNP and MNP-d_9

- **Each** line in the EPR spectra from MNP adducts is broadened by hyperfine from the 9 equivalent protons on the spin trap



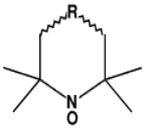
Spin labels

- If compound of interest does not have an unpaired electron it has no ESR.
- Attach (by reaction) a compound (spin label) that does; its ESR spectrum will hopefully change reflecting the new environment that the spin label is in.
- *Piperidinyloxy free radical*



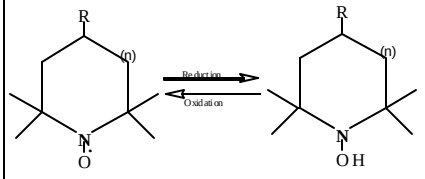
Spin labels

- **Nitroxide radicals give strong signals, and can be stabilised by placing adjacent methyl groups**
- **Other probes are possible, such as Mn(II) {substitutes for Mg(II)}, other paramagnetic transition metal ions, lanthanide ions, and some other organic radicals.**



Spin labels

Nitroxides can provide redox status dependent contrast in MRI



n = 1, piperidine nitroxides eg. Tempol, Tempo, Tempone
n = 0, pyrrolidine nitroxides. Eg. Carbamoyl proxyl, carboxy proxyl

Nitroxide radical	**Hydroxylamine**
Paramagnetic	**Diamagnetic**
Provides enhancement in T1 based MRI	**Does not provide T1 contrast in MRI**

Analysis of Proteins

The Problem

- Although there are a majority of x-ray crystallographic results published which give insight into the three-dimensional structure of a protein, it is evident that techniques must be established that will correlate structure and dynamics directly to function.

Analysis of Proteins

The Technique

- Currently, two experimental techniques work cooperatively to give the desired information on large amplitude motions in proteins
- Site Directed Spin-Labeling (SDSL) followed by
- Electron Spin Resonance (ESR)

Analysis of Proteins

Site Directed Spin Labeling

- Paramagnetism is essential

  -When using ESR to perform analysis the sample must be paramagnetic.

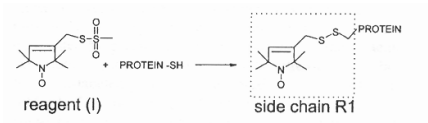
  -Stable proteins are not paramagnetic.
- How is this problem fixed?

  -A paramagnetic Nitroxide “spin-label” is introduced into the protein through site directed mutagenesis, in this way ESR can detect signals from the nitroxide and distance information can be extracted

Analysis of Proteins

Site Directed Mutagenesis

The General Mechanism is as follows:



(2)

A protein with a site-specific cysteine mutant is first created. Once this task is accomplished, the addition of a methanethiosulfonate reagent is required to attach the nitroxide.

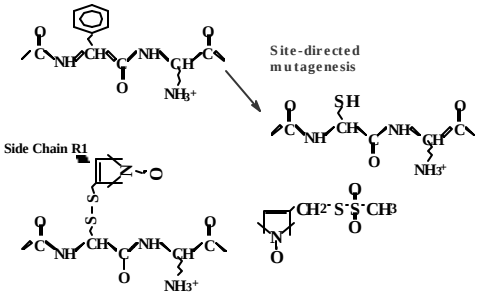
Analysis of Proteins

Site Directed Spin Labeling

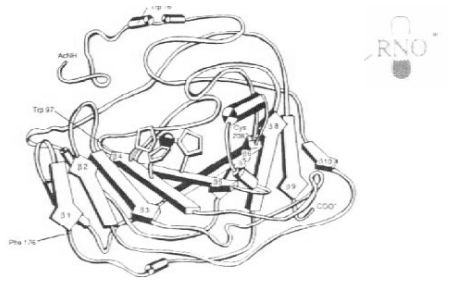
- Once the nitroxide has been introduced to the system, ESR analysis can be performed.
- Often, two nitroxide spin labels are attached at different sites in the protein to obtain distance measurements with the ESR.

Analysis of Proteins

Site-Directed Spin Labeling



Spin Labels



What do we do with EPR?

We can detect & measure free radicals and paramagnetic species

- High sensitivity (nanomolar concentrations)
- No background
- Definitive & quantitative

Direct detection

primary species, detected as is
e.g.: semiquinones, nitroxides, trityls

primary species are detected intact as spin-adduct, 'spin-trapping'
Species: superoxide, hydroxyl, alkyl, NO
Spin-traps: DMPO, PBN, DEPMPO, Fe-DTCs

Indirect detection (secondary radicals)
Spin-formation : hydroxylamines
Spin-change : nitronylnitroxides
Spin-loss : trityl radicals

Can we use EPR to measure free radicals from biological systems?

(in vivo or ex vivo)

Yes! Radicals from intact tissues, organs or whole-body can be measured.

But there is a catch!

Biological samples are aqueous and undergo 'non-resonant' absorption of microwave energy (microwave cooking!) and hence poor penetration depth.

The frequency of the instrumentation is reduced to overcome this problem!

What is the optimum frequency? - depends on sample size

Frequency	~300 MHz	~750 MHz	1-2 GHz	~3 GHz	9-10 GHz
Penetration Depth	> 10 cm	6-8 cm	1-1.5 cm	1-3 mm	1 mm
Objects studied	Mouse, rat	Mouse	Mouse, rat heart	Mouse tail Topical (skin)	In vitro samples (~100 uL vol.)

What else can we do with EPR?

Instead of "spying on free radicals", we can use free radicals as "spying probes" to obtain functional information from biological systems

- A known free radical probe is infused or injected into the animal
- The change in the EPR line-shape profile, which is correlated to some physiological function, is then monitored.
- The measurements can be performed in *real-time* and *in vivo* to obtain 'functional information'.

Summary

- The main feature of EPR spectra that is useful for assignment to a particular free radical structure is hyperfine splitting
- Direct EPR spectra can provide a wealth of structural information
- Highly unstable free radicals can, in many cases, be stabilized for EPR characterization by spin trapping
  - The increased stability of the detected free radical comes with a loss of structural information
  - The adduct may undergo chemistry between formation and detection
  - Adduct assignment is assisted by selective isotope labeling and EPR analysis of an independent preparation of the suspected adduct
  - The performance of appropriate controls is essential