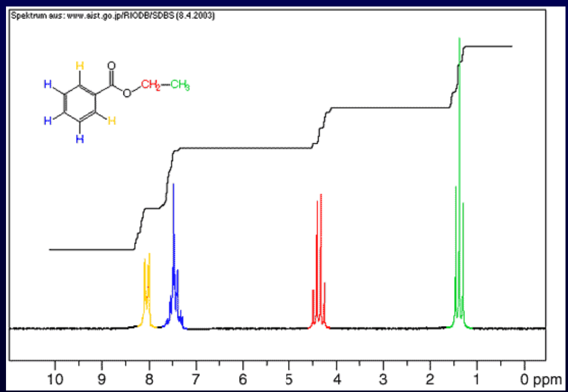
Magnetic Resonance Spectroscopy (EPR, NMR) as structural tools

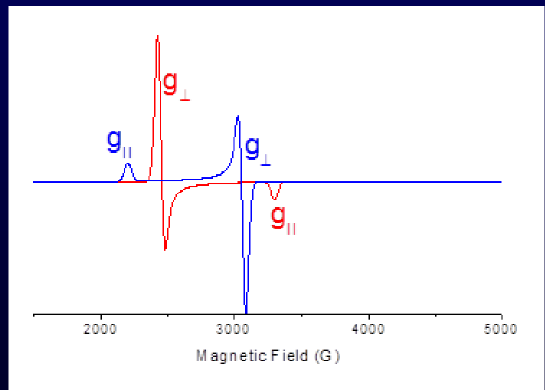
Hendrik Küpper, Advanced Course on Bioinorganic Chemistry & Biophysics of Plants, summer semester 2025
based on a lecture of Peter Kroneck, Universität Konstanz

Introduction: EPR and NMR

Prof. Richard Ernst,
one of the fathers of
biological NMR

Prof. Helmut Beinert,
one of the fathers of
biological EPR

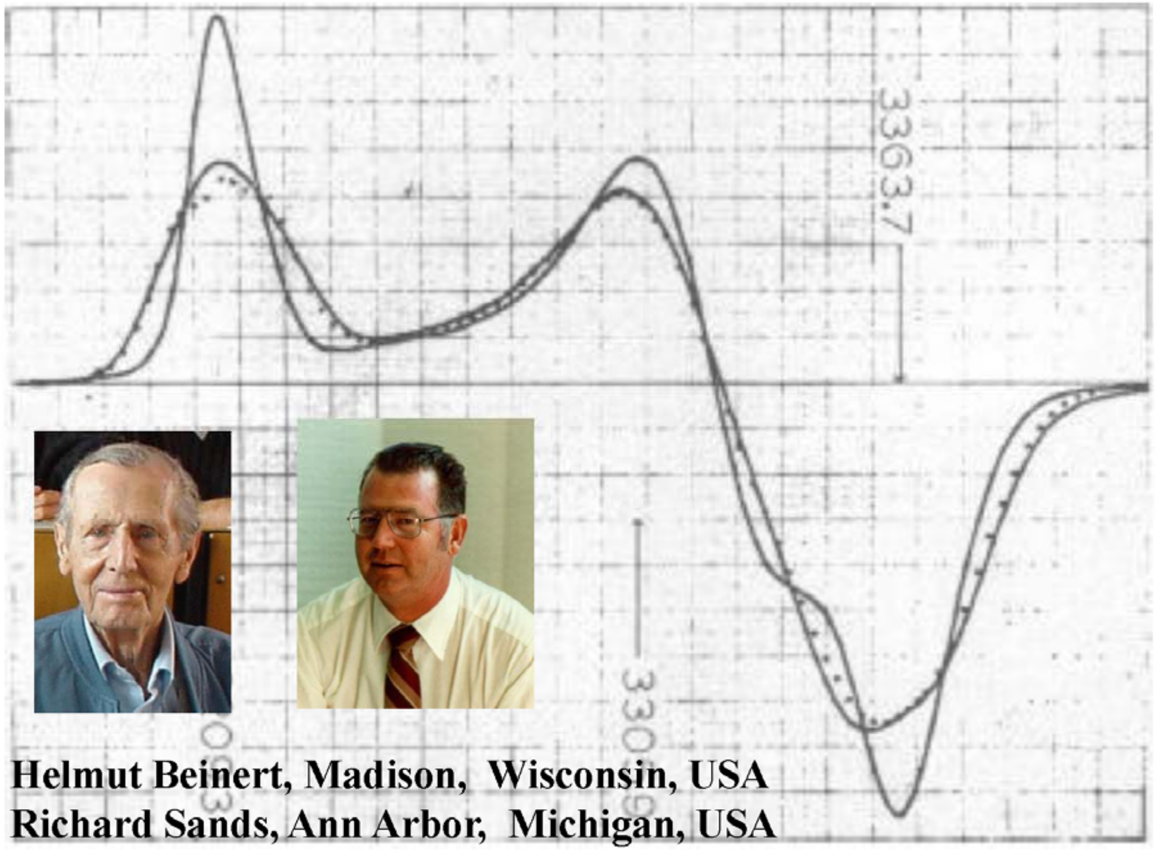




→ Electron Paramagnetic Resonance and Nuclear Magnetic Resonance are non-invasive techniques. They can be applied to living systems *in vivo* to obtain images, such as distribution of H_2O, O_2, or NO in tissues, or for analysis of molecular structures, also of metal complexes

History: The discovery of a new Iron Center

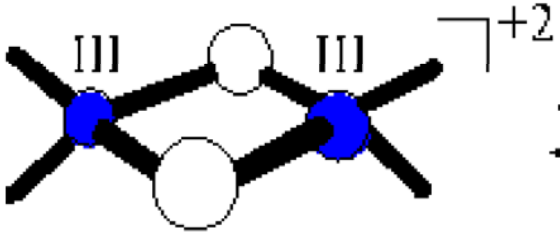
IDENTIFICATION BY ISOTOPIC SUBSTITUTION OF THE EPR SIGNAL AT g = 1.94 IN A NON-HEME IRON PROTEIN FROM *AZOTOBACTER,* Y I SHETHNA, P W WILSON, R E HANSEN, H BEINERT, Proceedings National Academy of Science/USA (1964), *52*, 1263-1271



Helmut Beinert, Madison, Wisconsin, USA
Richard Sands, Ann Arbor, Michigan, USA

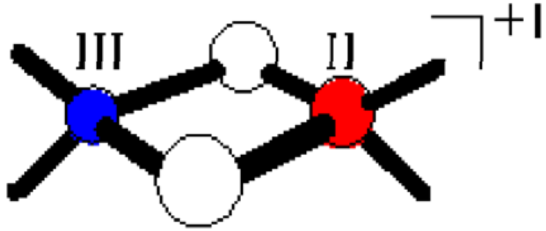
EPR spectra of Fe^{56} and Fe^{57} iron proteins superimposed. The dotted curve represents a computed curve for the Fe^{57} protein, which was obtained from the curve of the Fe^{57} protein, assuming a hyperfine splitting of 22 G and a final enrichment of 65% for Fe^{57}.

S = 0



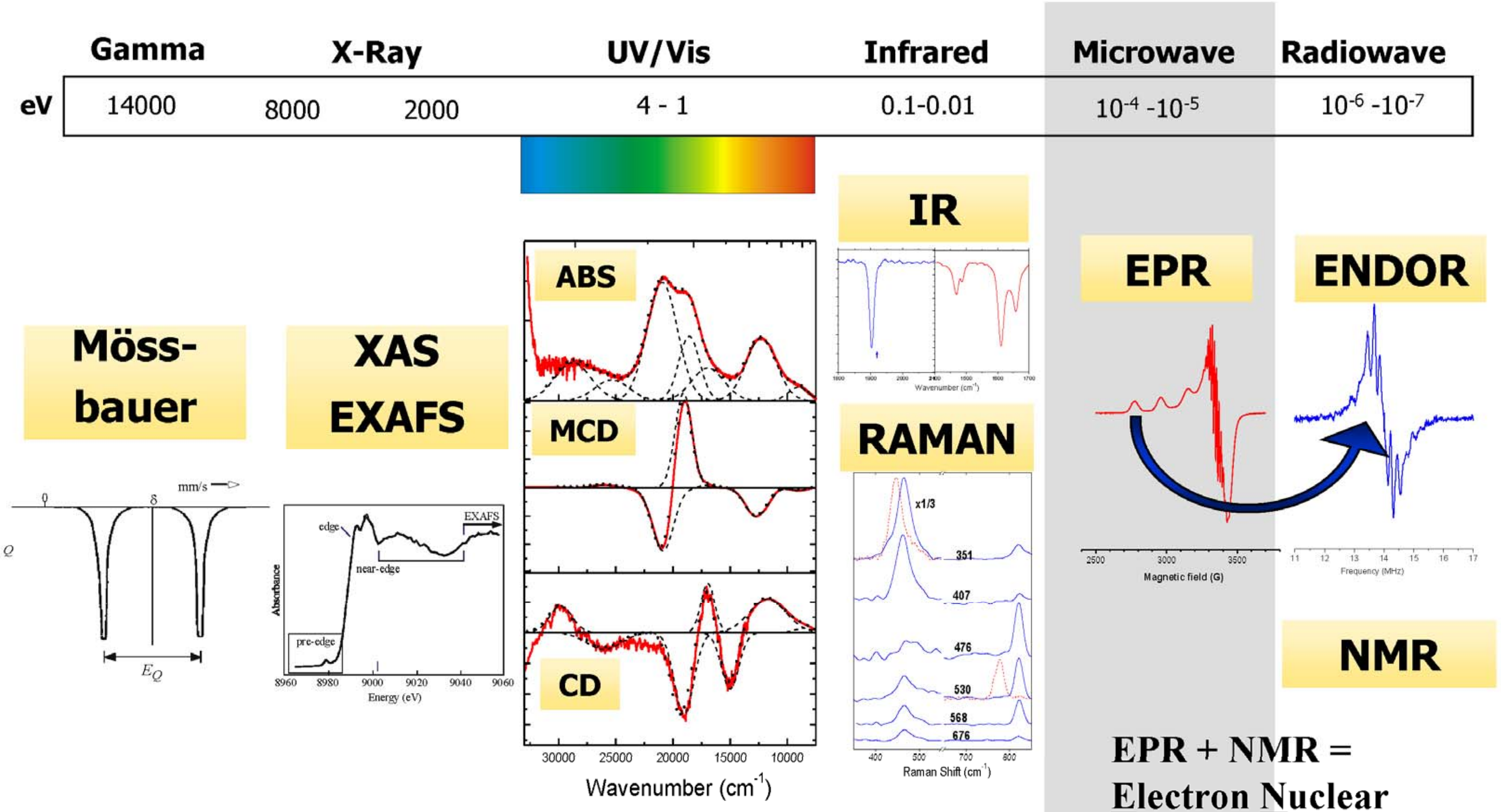
$+ e^-$

$\rightleftarrows$



S = 1/2

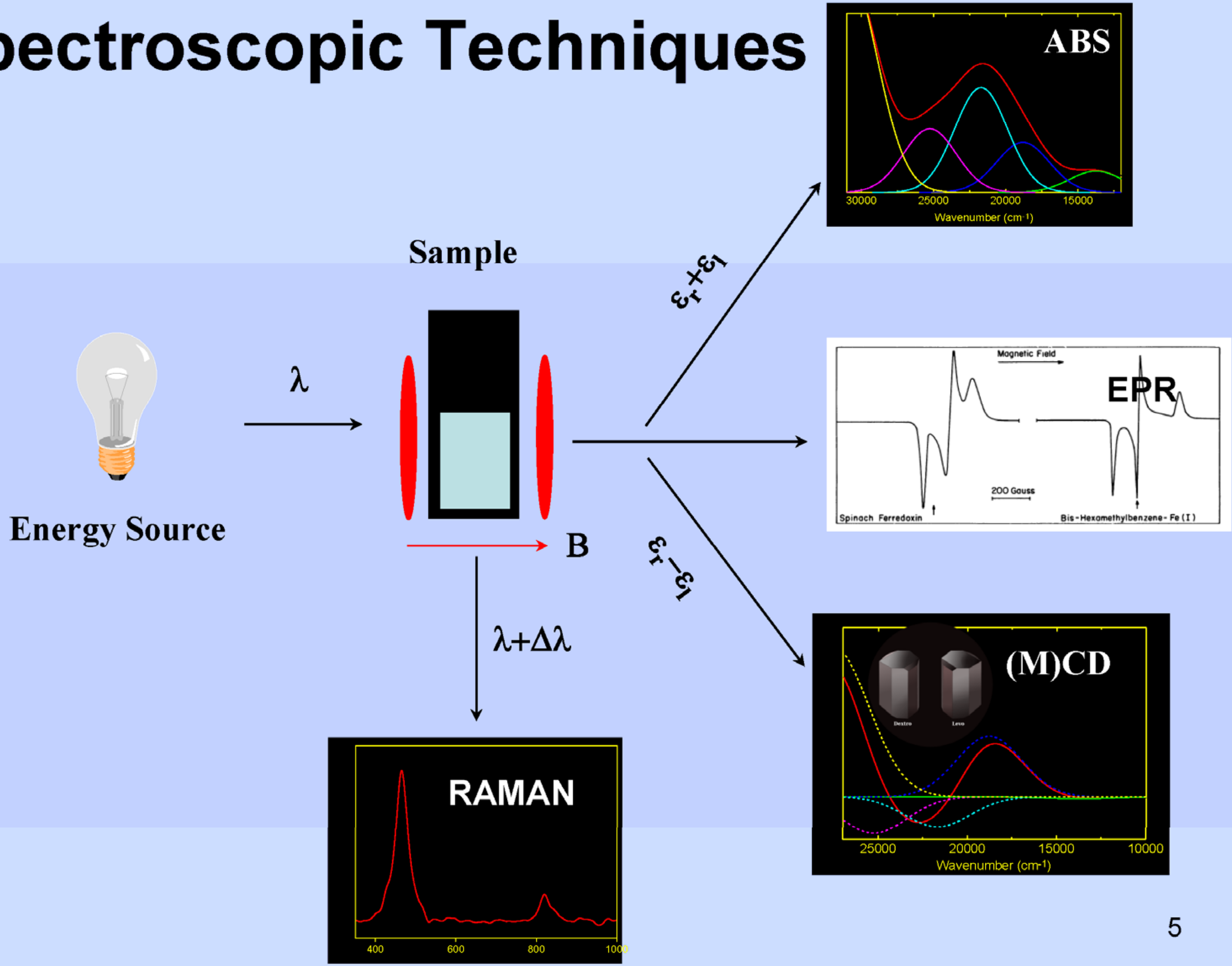
Spectroscopic Techniques - Energy



<u>C</u>ircular <u>D</u>ichroism
<u>M</u>agnetic <u>C</u>ircular <u>D</u>ichroism

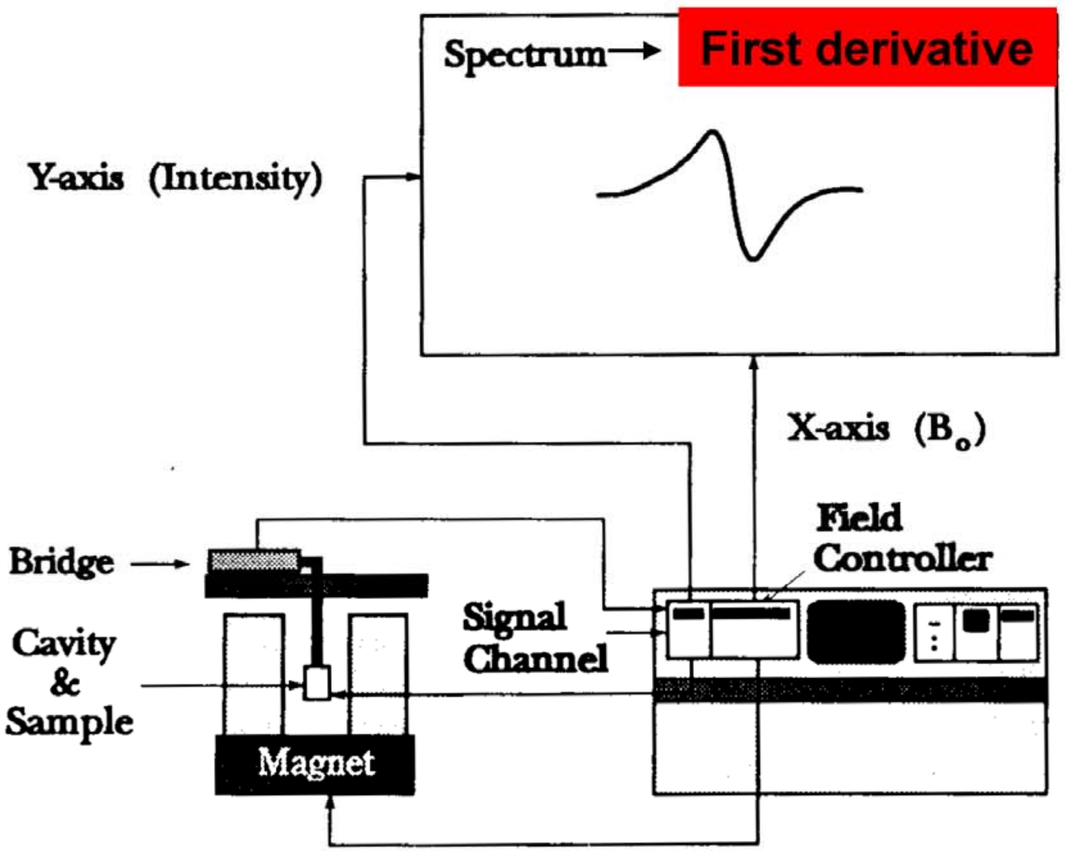
EPR + NMR =
<u>E</u>lectron <u>N</u>uclear
<u>Do</u>uble <u>R</u>esonance

Spectroscopic Techniques

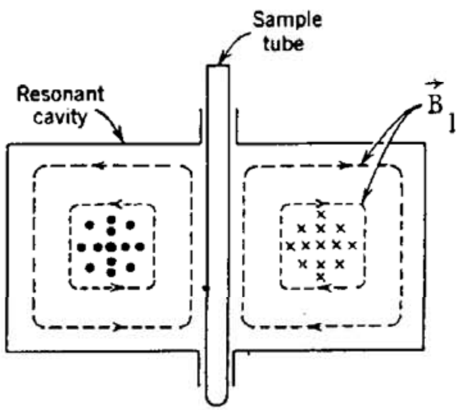


Commercial CW-EPR Instrument

sample in 3-4 mm quartz tube; vol. 250 µl; conc. “as much as possible” (0.1 – 0.01 mM)



Bridge=„Microwave lamp“



Sample Cavity

Important: Cryotechnology/Variable Temperature
Depending on the metal ion liquid N_2 (77K) or He (4.5K)

Cryostat

EPR - Basic Information

1. **Is the substance paramagnetic ? (Oxidation state of metal ion)**
   Note: *Integer Spin Systems* might be EPR silent/Technology !
2. **Which type of paramagnet is present ?**
   Fingerprinting ! Metal, Organic Radical, Interacting systems
3. **How much paramagnet is present ?**
   Quantification !
4. **Geometric and electronic structure of paramagnet**
5. **Information about type and number of ligands**
6. **In interacting systems, information about distances**

Applications of EPR

Which compounds can be studied by EPR ? Radicals

Paramagnetic systems with unpaired electrons, S≠ 0

In Inorganic Biological Chemistry, Biology, and Medicine

1. **Most transition metals: Cu^{II},$Ni^{I,III}$,Co^{II},Fe^{III},$Mn^{II/III/IV}$,V^{IV},Mo^{V}, W^{V}**
2. **Protein side chain radicals (Tyr•,Trp•,Gly•,Cys•)**
3. **Radical states of cofactors (Semiquinones, Flavins ...)**
4. **Inorganic radicals (NO•, O_2 , $O_2^{\bullet -}$, HO•....)**
5. **Transient species in light driven processes**

...but also

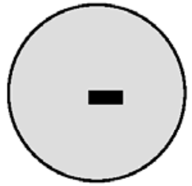
1. **Spin Traps can be used to catch short-lived radicals**
2. **Spin Labels can be attached to proteins, nucleic acids, ... to study their structure and dynamics**

Basic Properties of Electrons

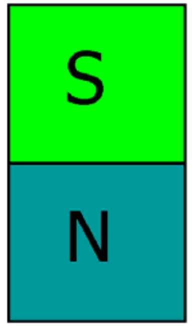
An Electron has the following properties:

- **Mass m_e**
- **Charge $-e_0$**
- **Spin $\rightarrow$ Magnetic Dipole Moment μ**

$$\mu = -\underbrace{g_e}_{2.002319\ldots}\ \underbrace{\beta}_{\text{Bohr's Magneton}}$$

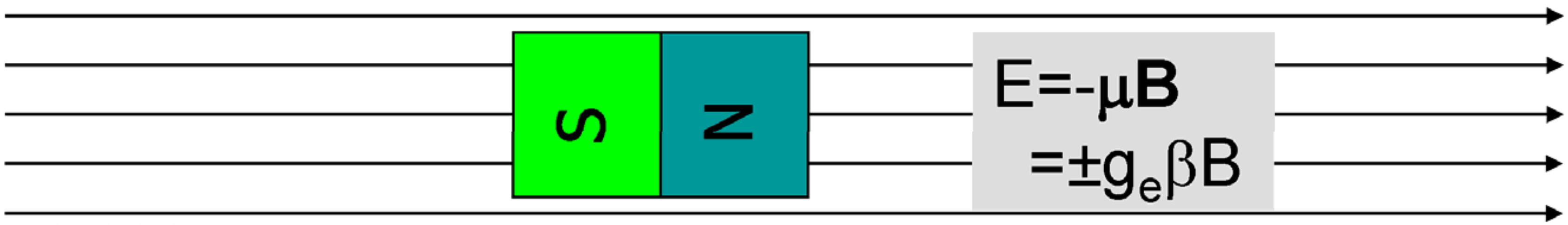


Electric monopole



Magnetic Dipole

The Magnetic Dipole in a Magnetic Field:



Field **B**

An Electron in a Magnetic Field

Energy of an Electron in a Magnetic Field:

$$E=-\mu\mathbf{B} = \pm g_e\,\beta\,|B|\cos(\theta)$$

In Quantum Mechanics:

Only Orientations with $\cos(\theta)=\pm 1/2$ are possible

Thus, the Electron can have only two states:

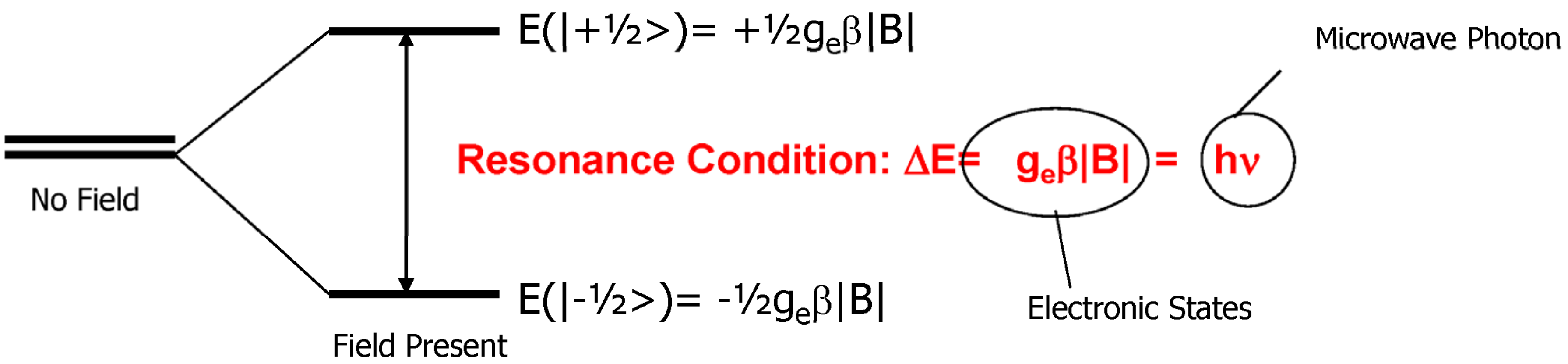
$|+1/2\rangle$ and $|-1/2\rangle$

„up" **„down"**

The EPR Transition

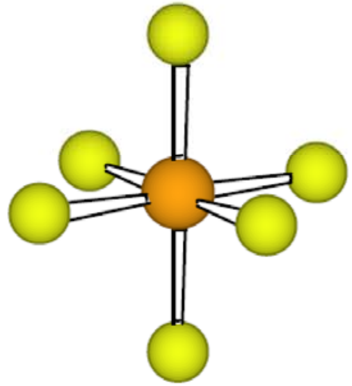
In order to change the orientation of the electronic magnetic dipole moment in the presence of a magnetic field we need to apply a *FORCE*.

A suitable force is provided by a microwave photon which induces a transition between the |-½> and |+½> levels.



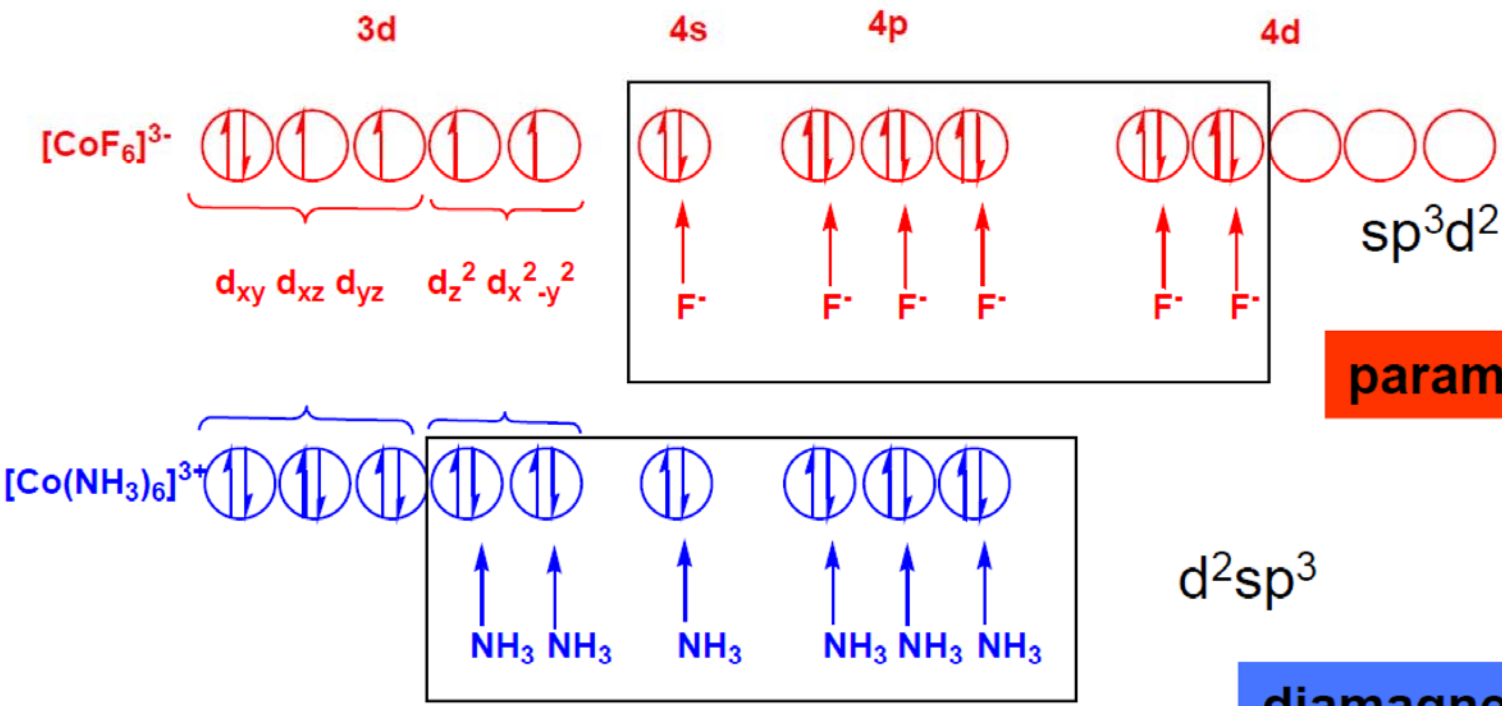
Remember: Valence Bond Theory

L. Pauling



$[Co(NH_3)_6]^{3+}$ and $[CoF_6]^{3-}$

d^6



sp^3d^2

paramagnetic, S=2

d^2sp^3

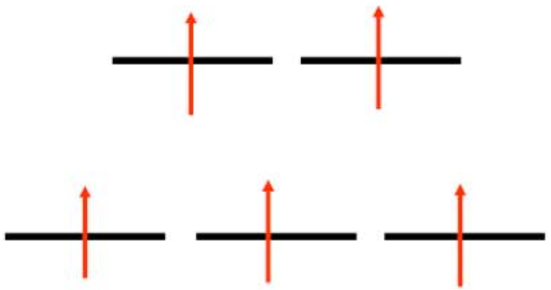
diamagnetic, S=0

sp^3d^2 and d^2sp^3 hybridization

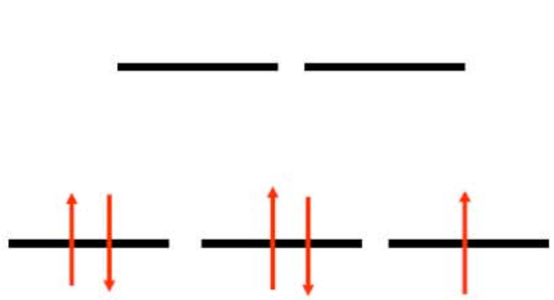
Color and Magnetism

Variable Spin States of Metal Centers

For a d^5 configuration, Fe(III)



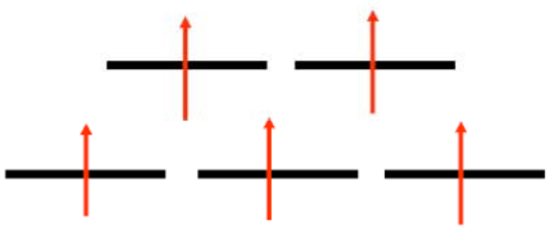
OR



High-Spin, S= 5/2

Low-Spin, S= 1/2

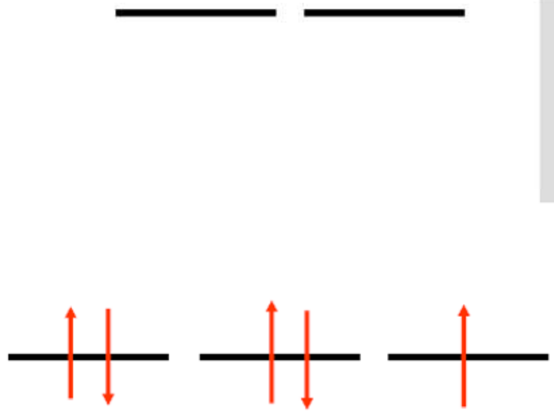
Depending on the METAL ION ENVIRONMENT, balance of Crystal Field Splitting, 10Dq and Spin-Pairing Energy, P



$10Dq < P$

Weak Field

High Spin

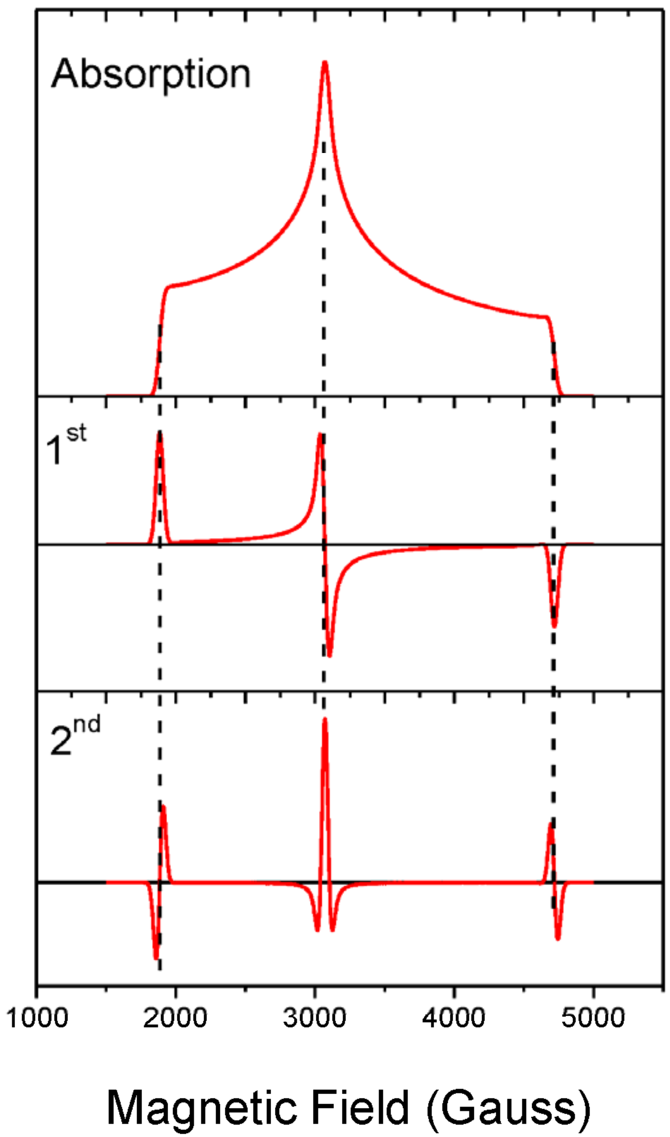


$10Dq > P$

Strong Field

Low-Spin

Presentation of EPR Spectra



The magnetic field is usually measured in <u>Gauss</u> (G) units. The SI unit, however, is the <u>Tesla</u> (T) !

$$1\,\mathrm{T} = 10\,000\ \mathrm{G}$$

$$1\,\mathrm{mT} = 10\ \mathrm{G}$$

Typical resonance field

$$B_{res} \sim 3000\ \mathrm{G} = 0.3\,\mathrm{T}$$

Multifrequency EPR

In EPR we usually <u>FIX</u> the microwave frequency ν (because of the cavity) and <u>VARY</u> the magnetic field B.

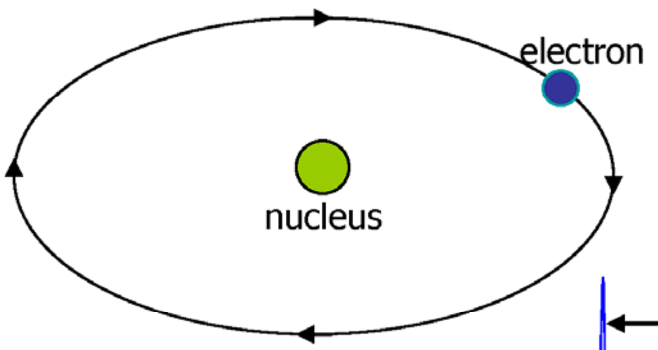
The magnetic field scale is inversely proportional to energy !

Thus, for every frequency we need a different Cavity, and we might have to change the magnet:

S-Band	: 1-2 GHz
C-Band	: 2-4 GHz
X-Band	: 9-10 GHz (Standard)
Q-Band	: 35 GHz
W-Band	: 95 GHz
High-Field	: 100-600.. ? GHz

$g_{effective}$ / g_{eff} - bound electrons (atom, molecule)

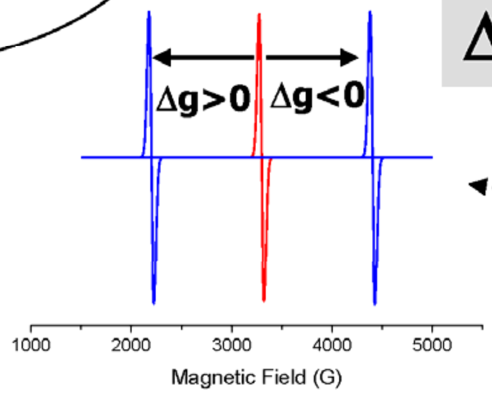
Resonance does not always occur at the same field: bound electrons carry some ANGULAR ORBITAL MOMENTUM *L* in addition to the SPIN ANGULAR MOMENTUM *S*.



Additional magnetic moment μ_L

Modification of Resonance Condition:

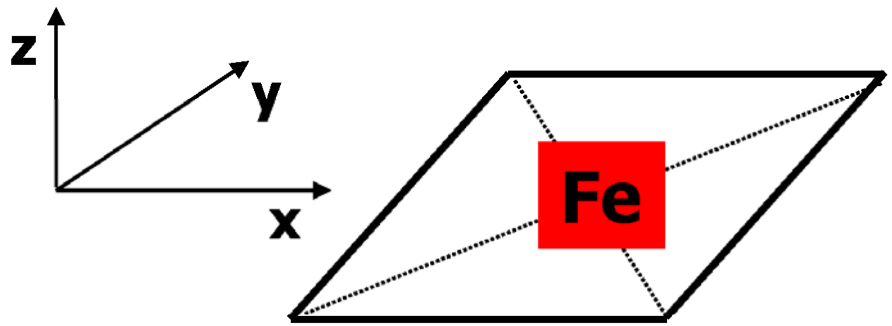
$$\Delta E = h\nu = -B(\mu_e + \mu_L) = \beta|B| \, g_{eff}$$



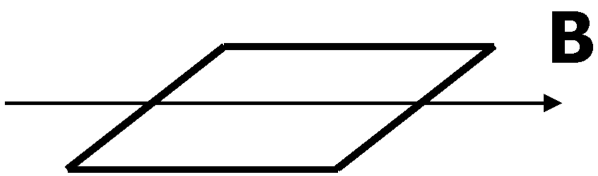
MOLECULAR Quantity=$g_e + \Delta g$

Anisotropy of g

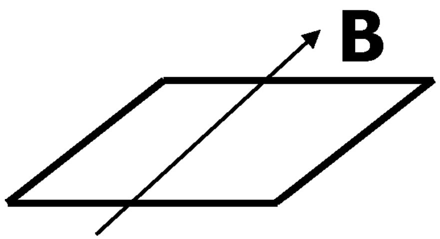
The relative orientation of B and $\mu = \mu_e + \mu_L$ matters a lot !



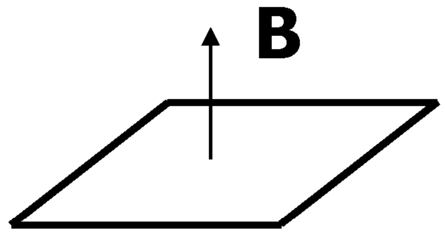
Consider three extreme cases:



$h\nu = -B_x\mu_x = \beta B_x \mathbf{g_x}$



$h\nu = -B_y\mu_y = \beta B_y \mathbf{g_y}$



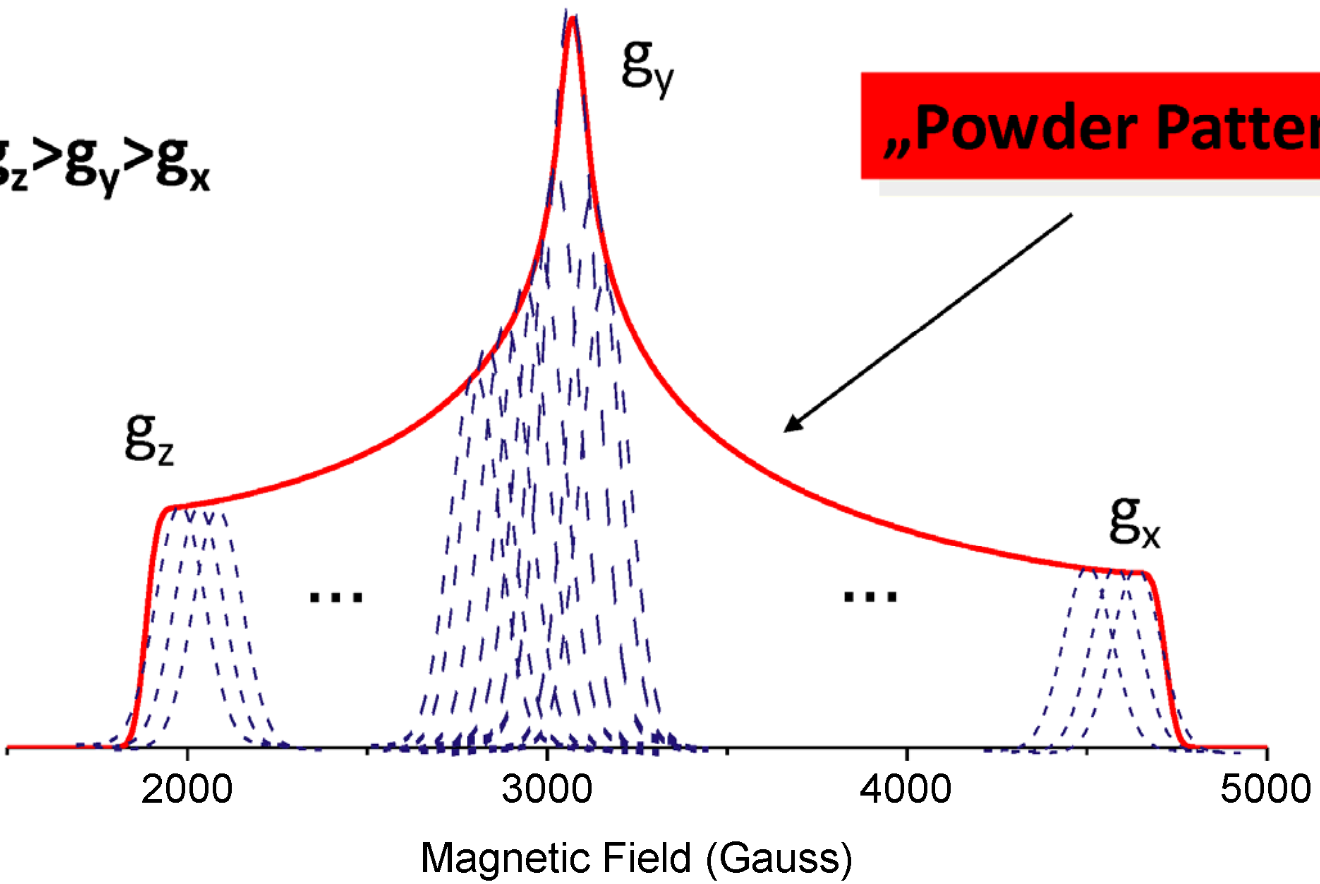
$h\nu = -B_z\mu_z = \beta B_z \mathbf{g_z}$

Thus, g becomes anisotropic: the „g-Tensor“

Consequence for the EPR Spectrum

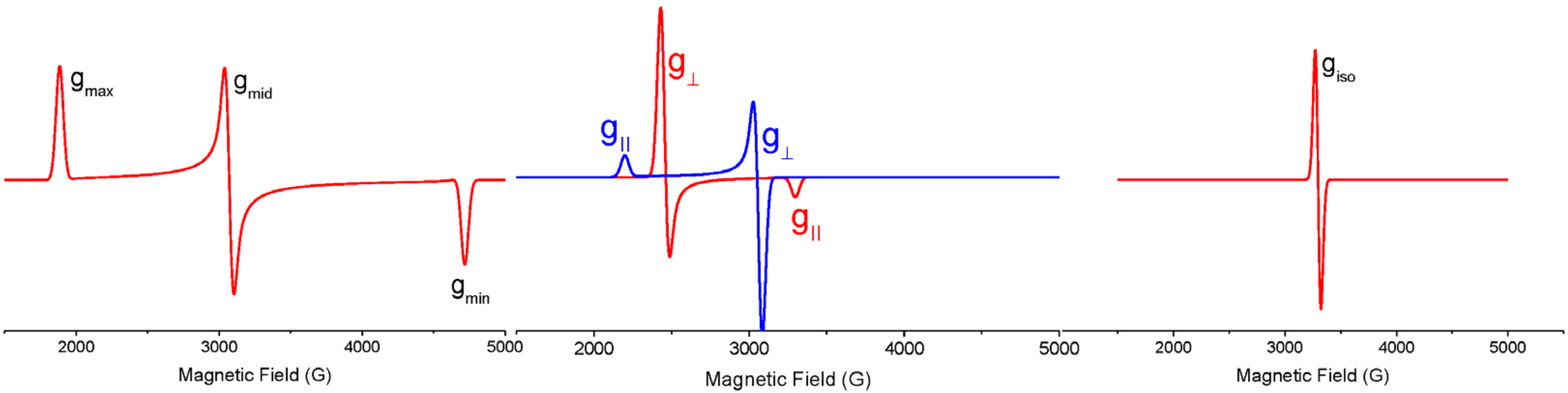
In Bio EPR we usually investigate frozen samples (randomly oriented molecules) and we have to integrate over all possible orientations !

Assume $g_z > g_y > g_x$



„Dialect“ for Powder Patterns

RHOMBIC	AXIAL	ISOTROPIC
$g_x \neq g_y \neq g_z$	$g_x = g_y (= g_\perp) \neq g_z (= g_{||})$	$g_x = g_y = g_z$

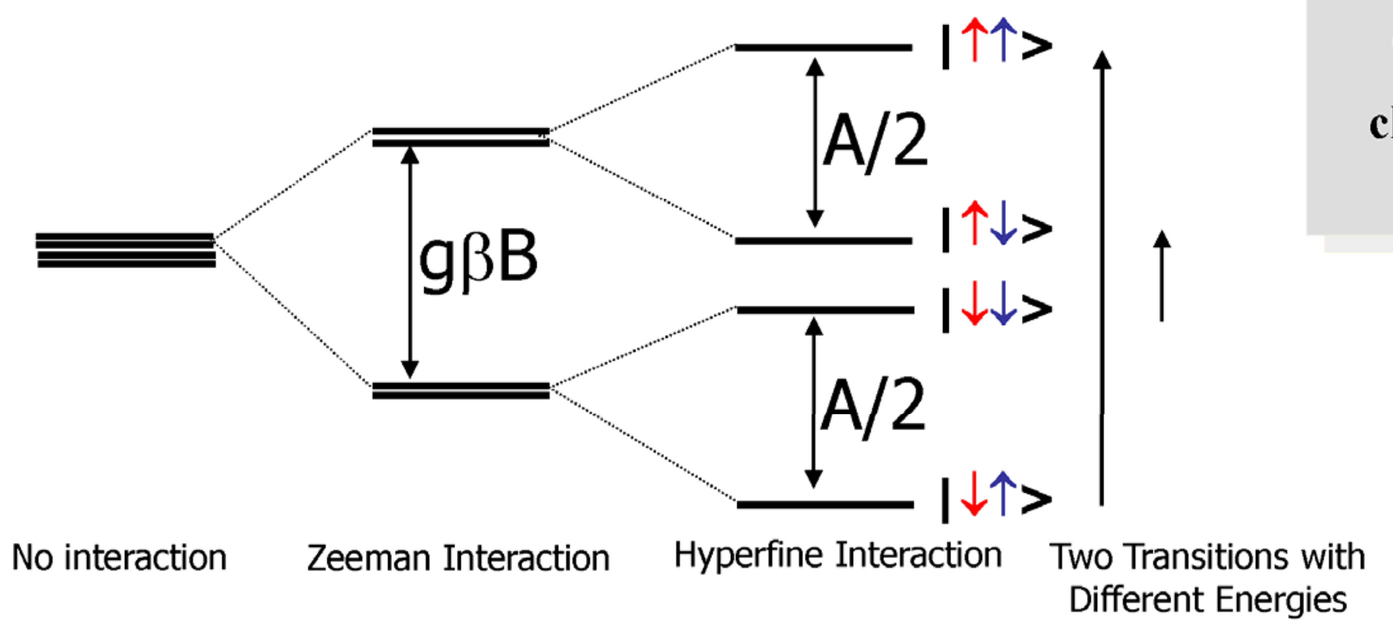


The Hyperfine Interaction (HFS)

Some Nuclei are *Little Bar Magnets* (→ NMR Spectroscopy)

- The condition is that the Nuclei have a Non-zero nuclear Spin I.

  ($^{1,2}H$, $^{14,15}N$, ^{17}O, ^{19}F, ^{33}S , ^{57}Fe, ^{61}Ni, $^{63,65}Cu$, ^{77}Se, ^{95}Mo, ^{183}W...)
- The Magnetic Interaction between the Nuclei and the unpaired Electrons is called Hyperfine Interaction (HFS, Symbol A)
- HFS leads to a Splitting of the EPR Lines

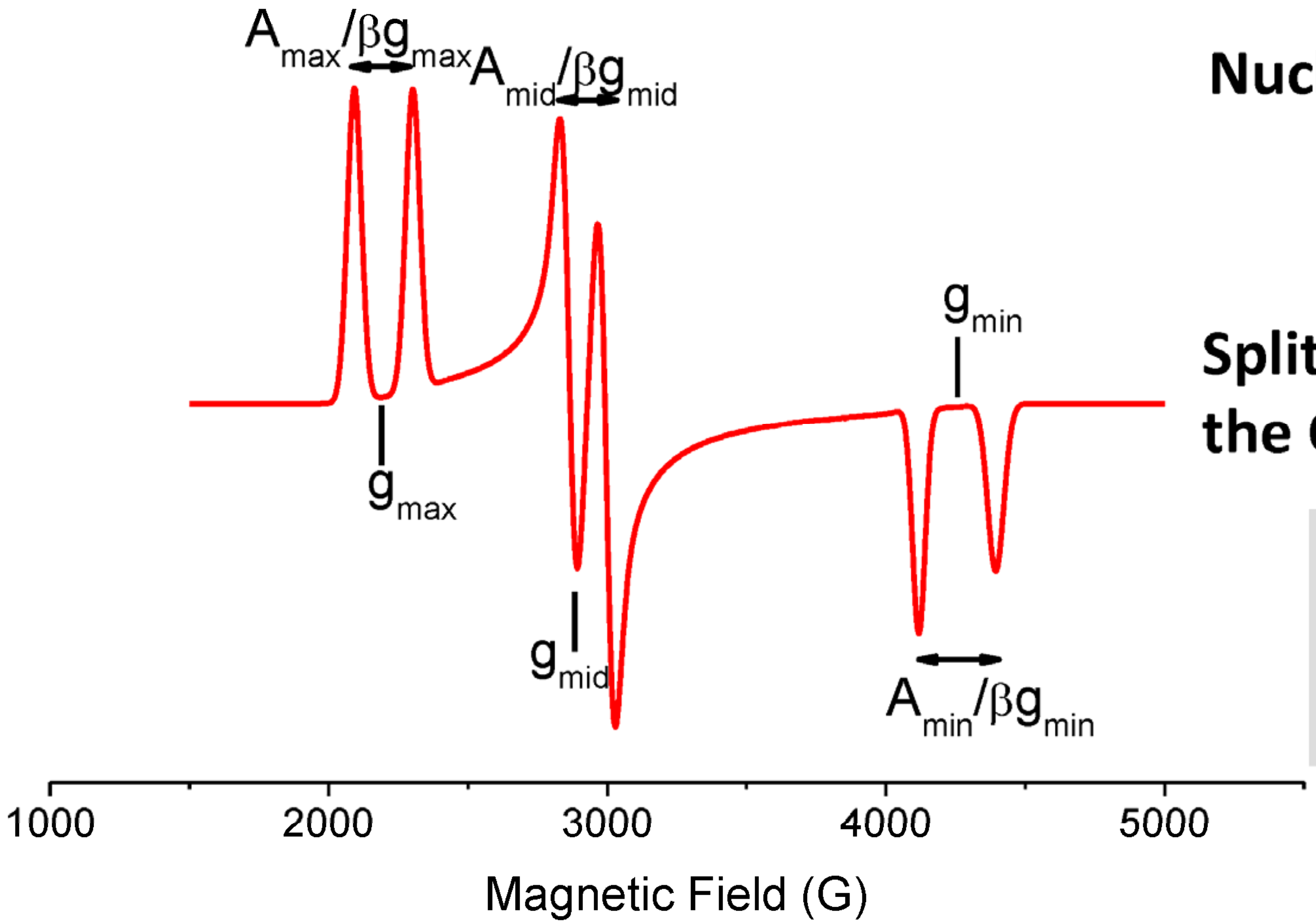


Selection Rule:
The Nuclear Spin does not change in an EPR Transition
$\Delta m_S = 1; \Delta m_I = 0$

↑ Electron Spin

↑ Nuclear Spin

EPR Spectrum with Hyperfine Structure



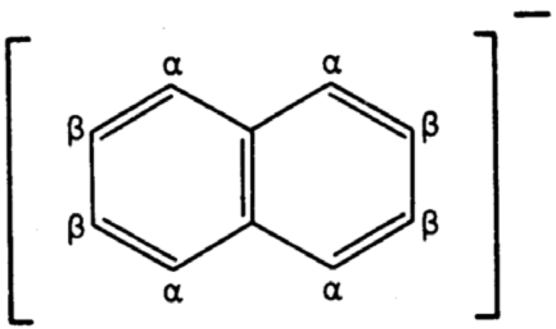
Nuclear Spin I = 1/2

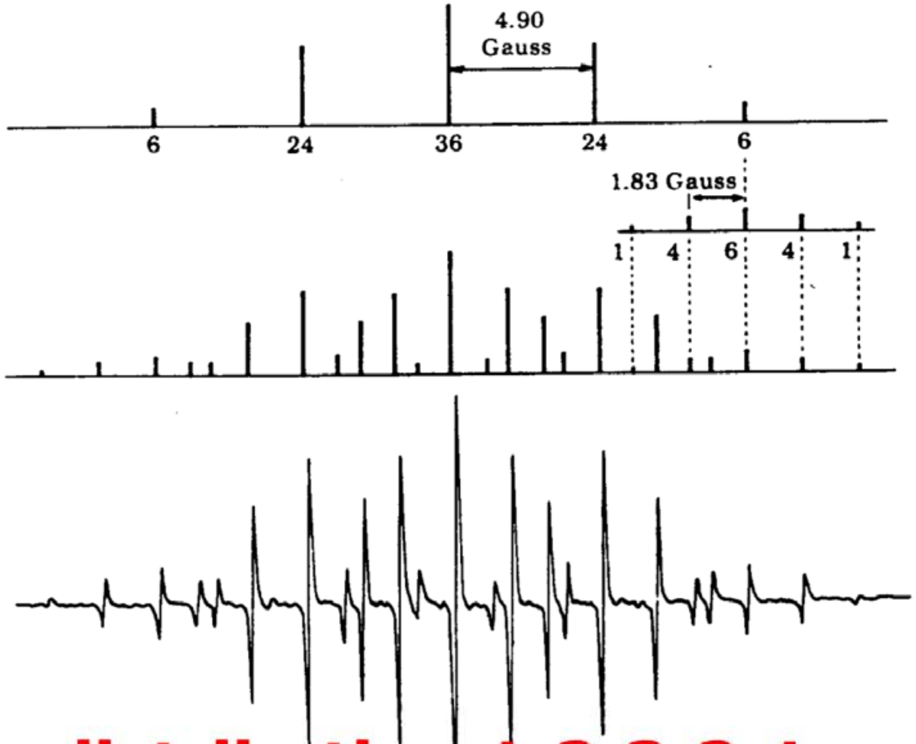
2I+1 Lines

Splitting Depends on the Orientation

A is different for each g-direction → „A-Tensor“

Study case 1: Naphthalene Anion Radical in solution; isotropic signal; HFS, $I^H = 1/2$





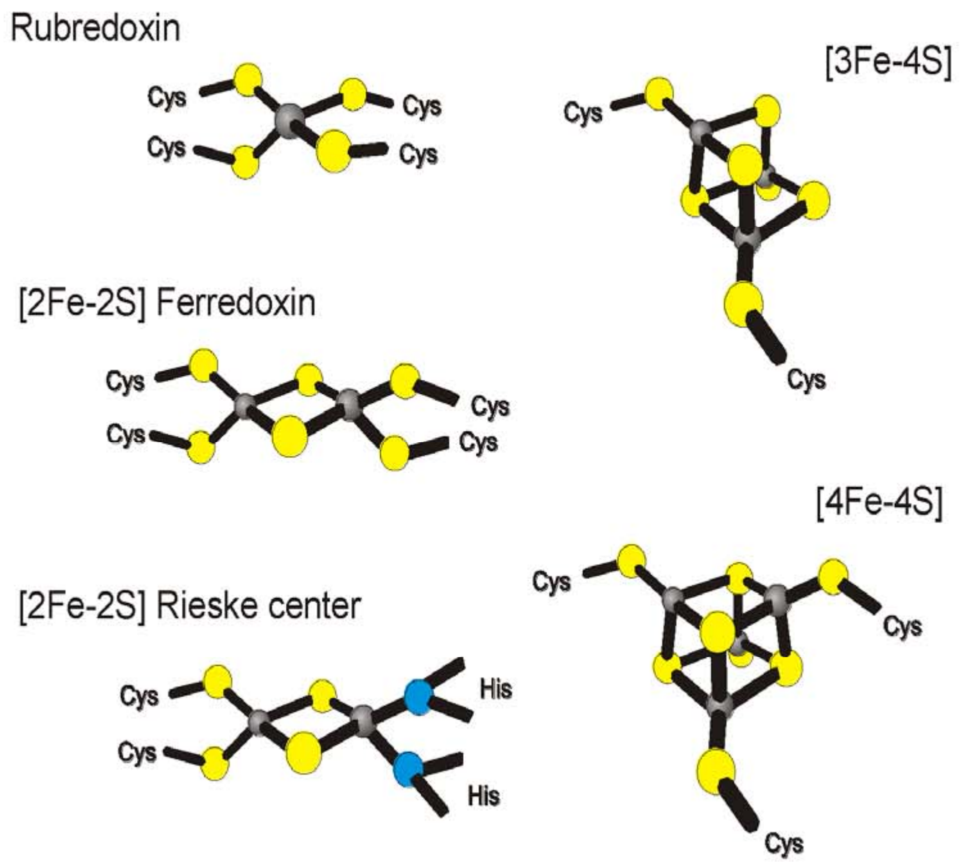
4 equivalent H = Intensity distribution 1:2:3:2:1

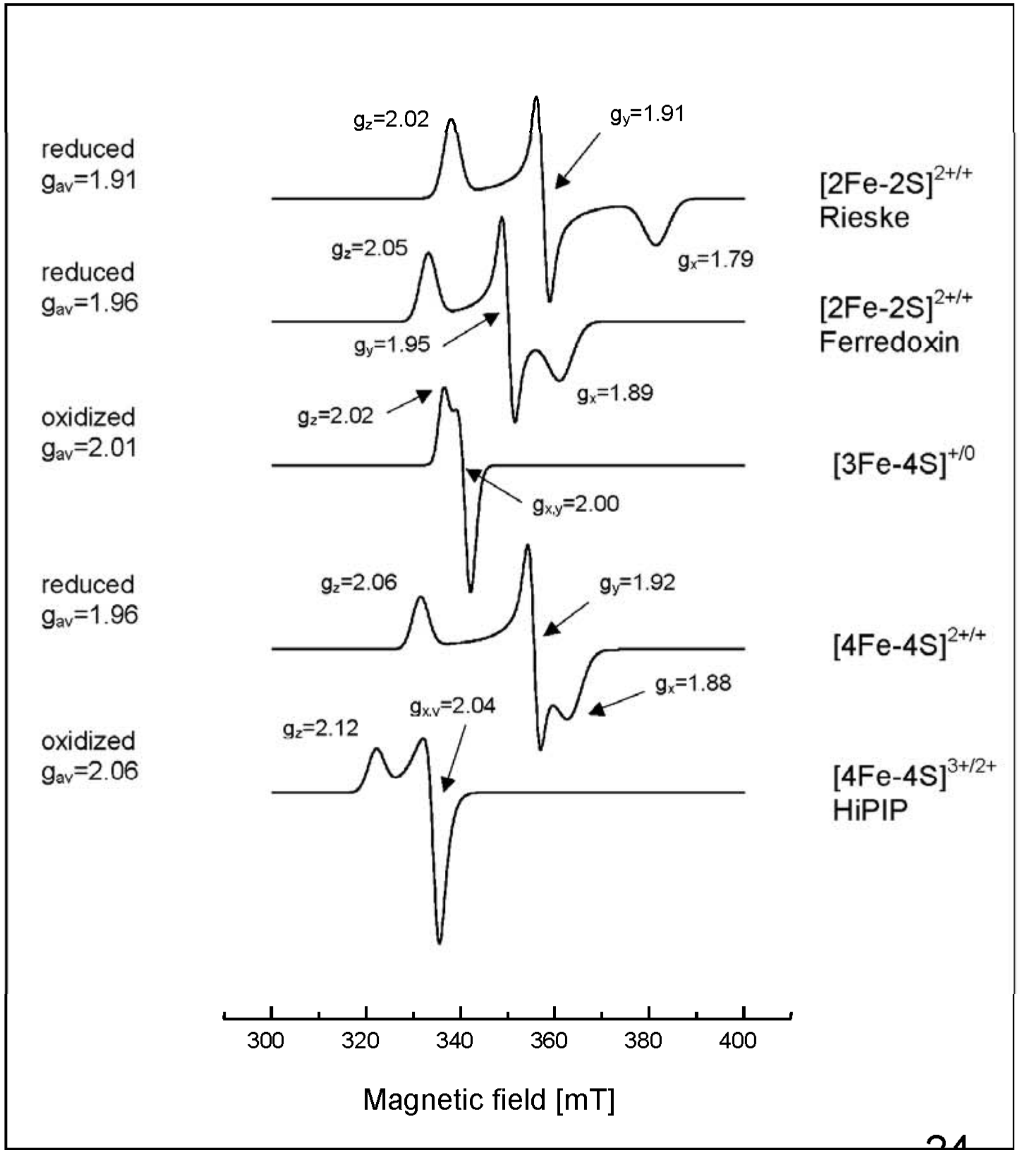
a_α=4.9 G

a_β=1.83 G

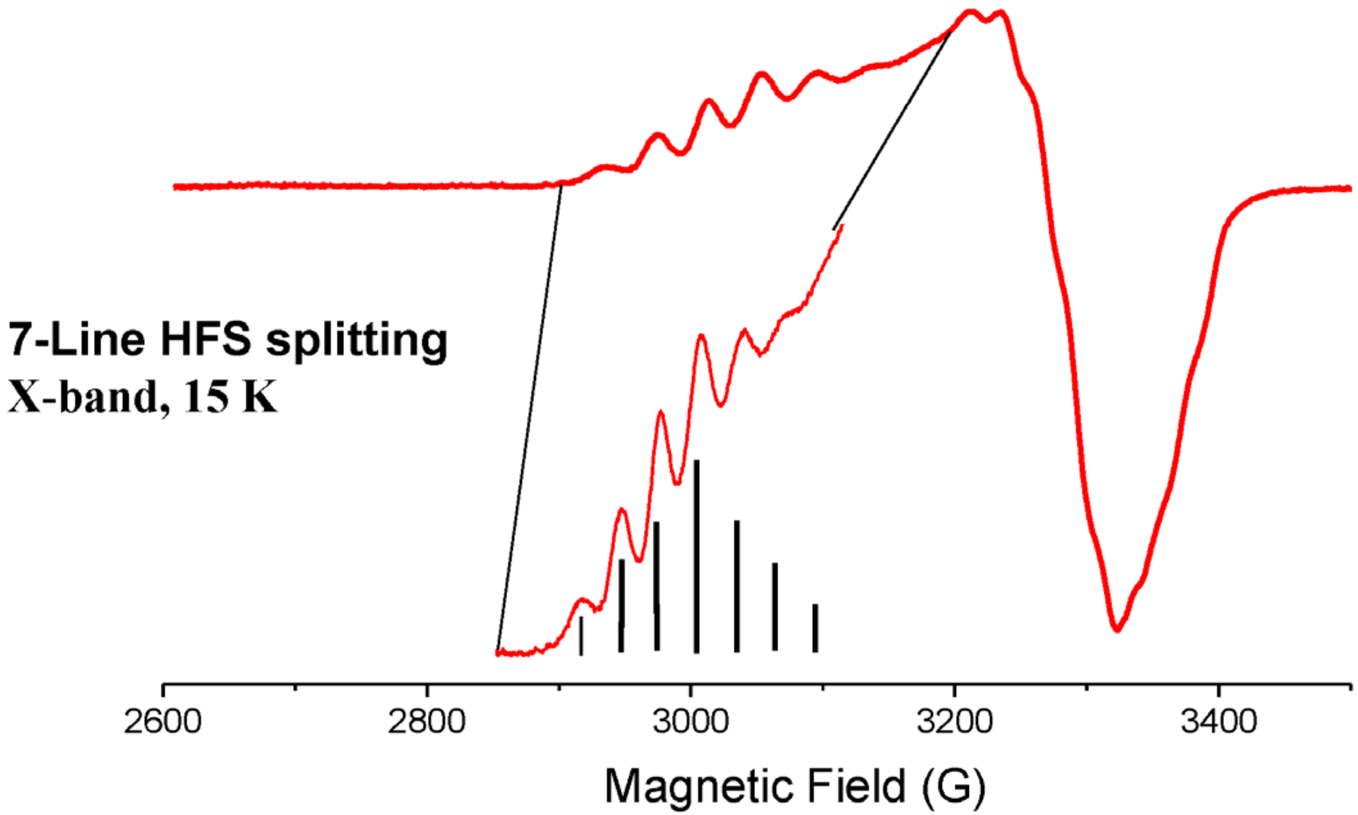
Note: Organic radicals usually have g-shifts which are very close to the free electron g-value g_e=2.002319

Study case 2: Iron–Sulfur (FeS) Centers frozen; 10K; anisotropic





Coyle CL, Zumft WG, Kroneck PMH, Körner H, Jakob W (1985)
Purple Nitrous Oxide Reductase. Eur. J. Biochem., **153**, 459-467



Neese F, Zumft WG, Antholine WE, Kroneck PMH (1996)
The purple Mixed-Valence Cu_A Center in Nitrous Oxide Reductase : EPR of the ^{63}Cu, ^{65}Cu and ^{65}Cu,^{15}N-histidine-enriched Enzyme and a Molecular Orbital Interpretation. J. Am. Chem. Soc. **118**, 8692-8699

Introduction: NMR

Nuclear Magnetic Resonance is a non-invasive technique which can be applied to living systems *in vivo* to obtain images, such as distribution of H_2O, O_2, or NO, in tissues. Furthermore, NMR spectra can be used for the following purposes:

→ Substance identification

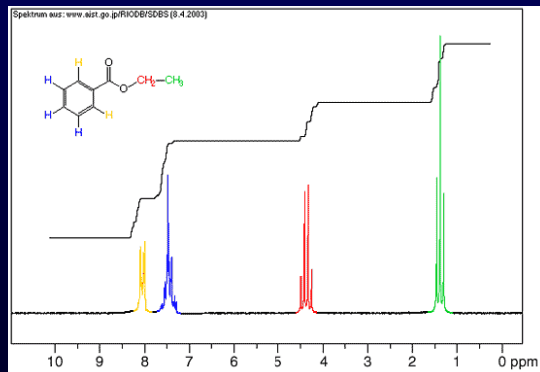
→ Purity

→ Fingerprint

→ Analysis of conformation

→ Determination of 3D structure (proteins, RNA, DNA)

→ Prof. Richard Ernst, one of the fathers of biological NMR



Configuration of a NMR magnet

Superconductive coil of wire in liquid helium (4 K)

Isolated by vacuum

Isolated by liquid nitrogen (77 K)

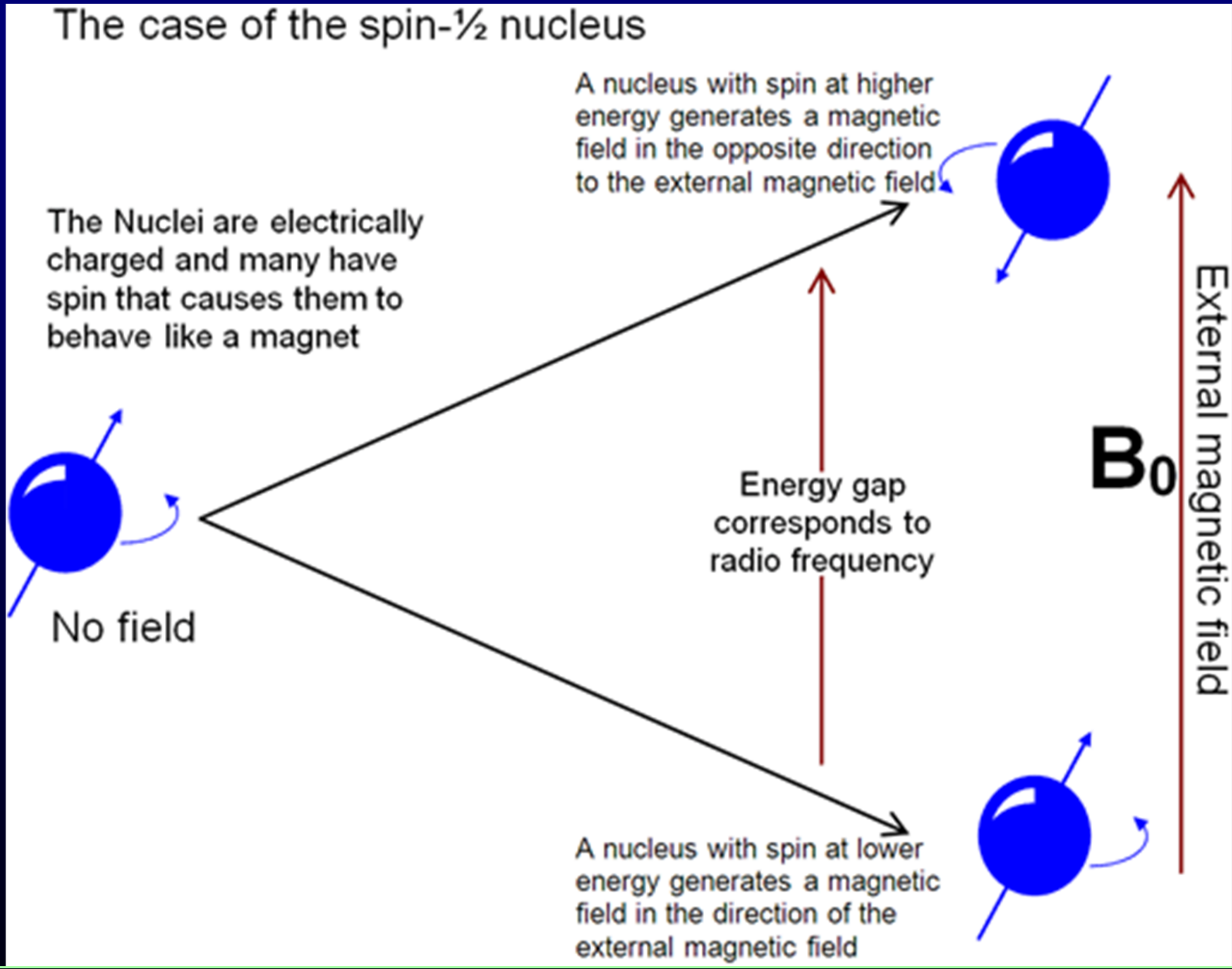
Again isolated by vacuum

NMR: Theory

Principle:

→ Nuclei with impair nuclear charges and impair mass numbers possess a spin, like electrons

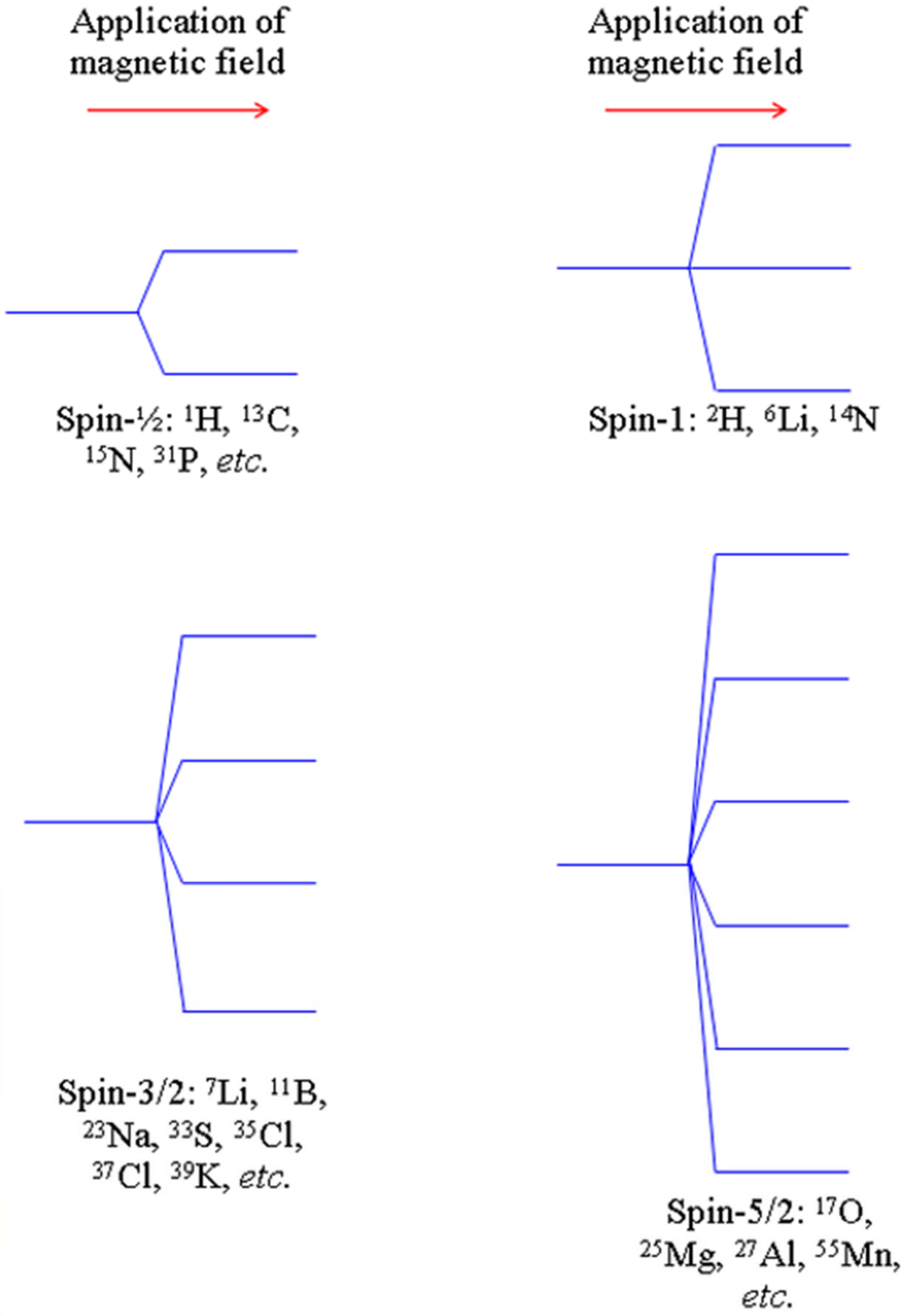
→ Application of an external magnetic field causes nuclei to behave differently depending on their spin



NMR energy levels of elements with spins >1/2

→ Most commonly studied nuclei: ^{1}H, ^{13}C, ^{15}N, ^{19}F, ^{31}P

→ Several metals are directly measurable as well, but requiring special techniques

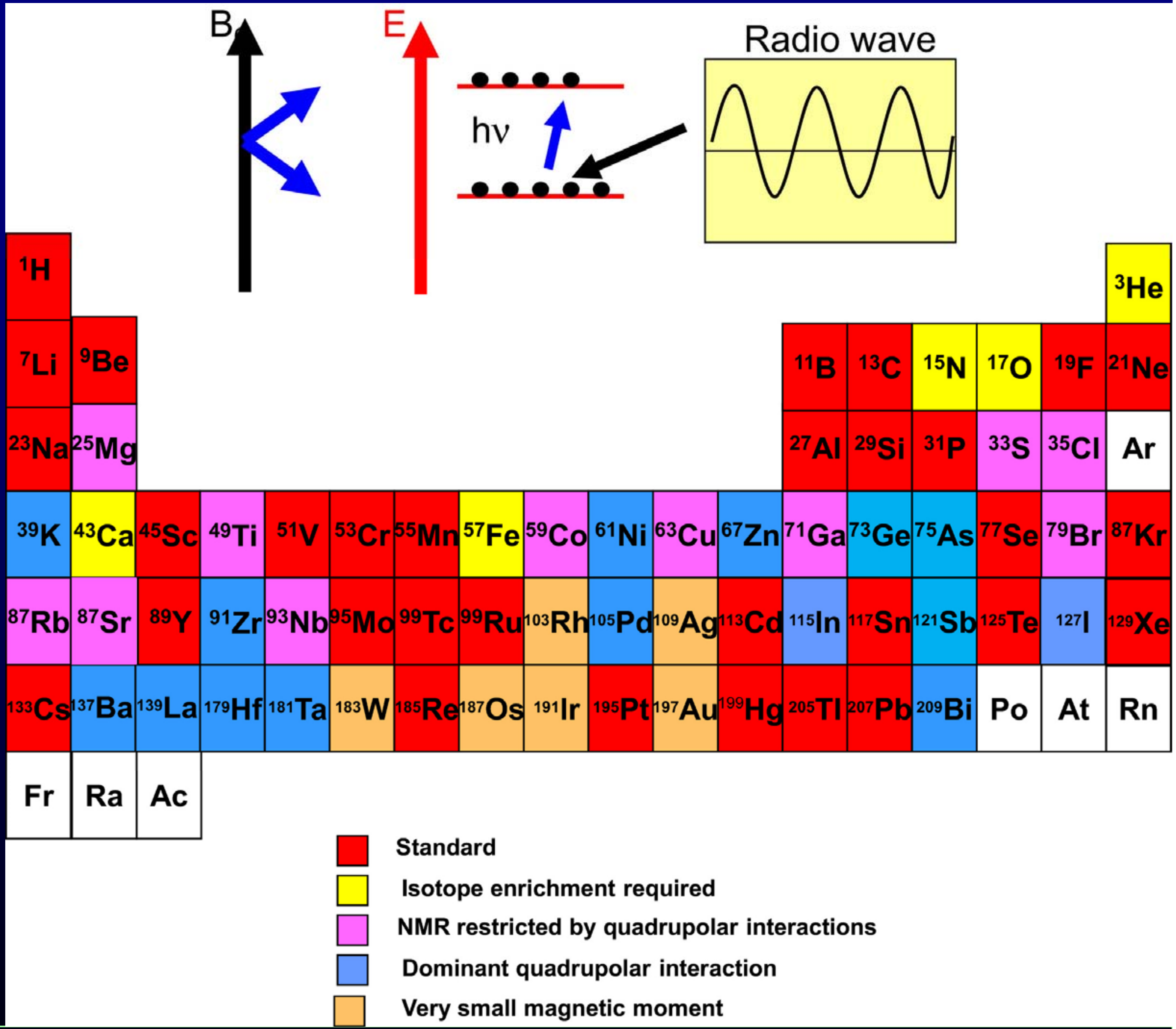


http://chem.ch.huji.ac.il/nmr/whatisnmr/quad_files/energylevels.gif

Elements measurable by NMR

→ Most commonly studied nuclei: ^{1}H, ^{13}C, ^{15}N, ^{19}F, ^{31}P

→ Several metals are directly measurable as well, but requiring special techniques

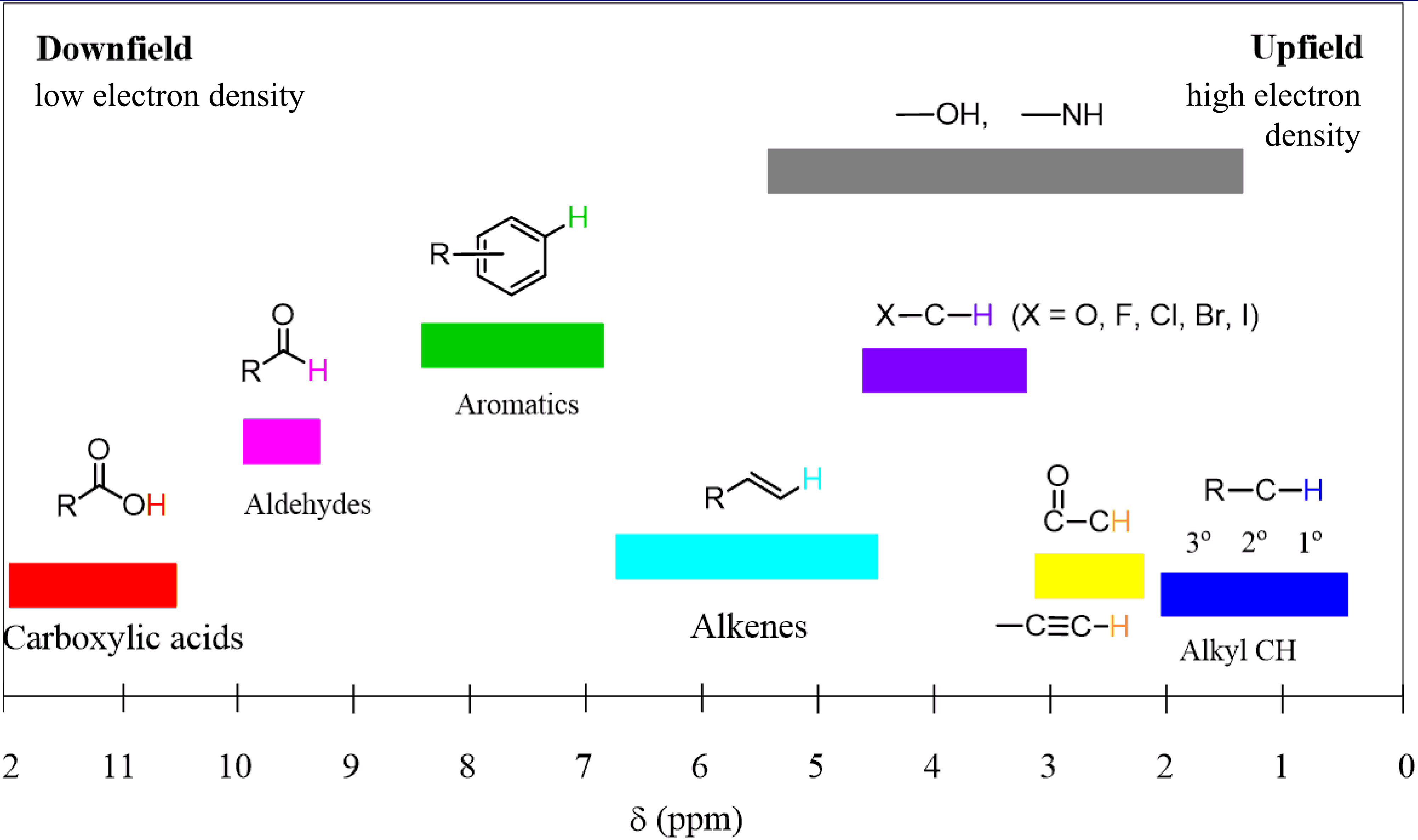


International Journal of Applied Glass Science, Volume: 9, Issue: 2, Pages: 167-187, 2017, DOI: (10.1111/ijag.12333)

NMR: Chemical shift

Principle: different functional groups cause the element measured to have different energy values for resonance absorption. Reference for chemical shift δ: Tetramethylsilan, Definition δ (TMS) = 0

Example: ^{1}H NMR (for heteronuclear NMR, the principle is the same)



https://www.chemistrysteps.com/wp-content/uploads/2020/01/NMR-ppm-signal-chemical-shif-values-for-proton.png

NMR: Number of interacting neighbouring nuclei

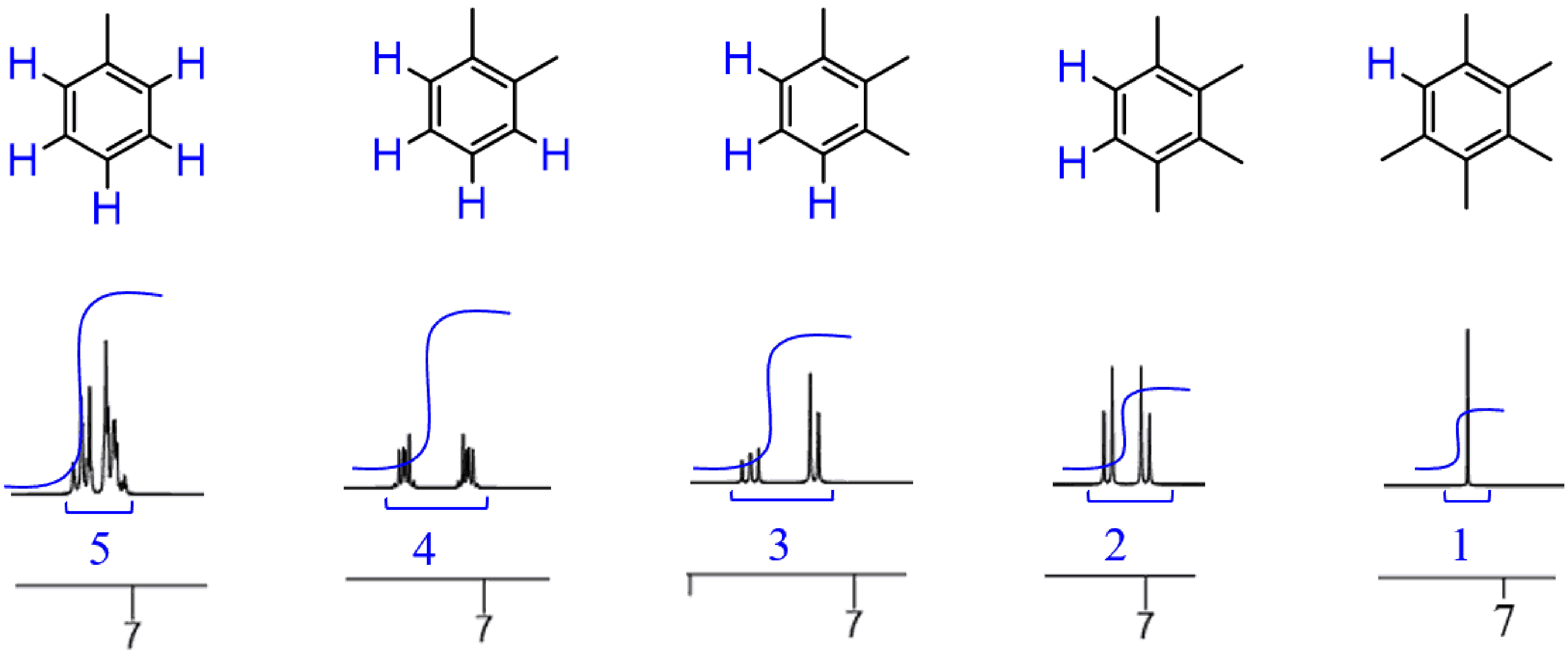
Principles

→ Neighbouring identical nuclei influence each other's NMR energy levels ("coupling"), causing multiplications of resonance energies if neighbourhoods of nuclei are different.

→ Coupling over at most 3-4 chemical bonds.

→ Integral of a resonance is proportional to number of neighbouring identical nuclei.

Example: ^{1}H NMR (for heteronuclear NMR, coupling cannot always be observed)

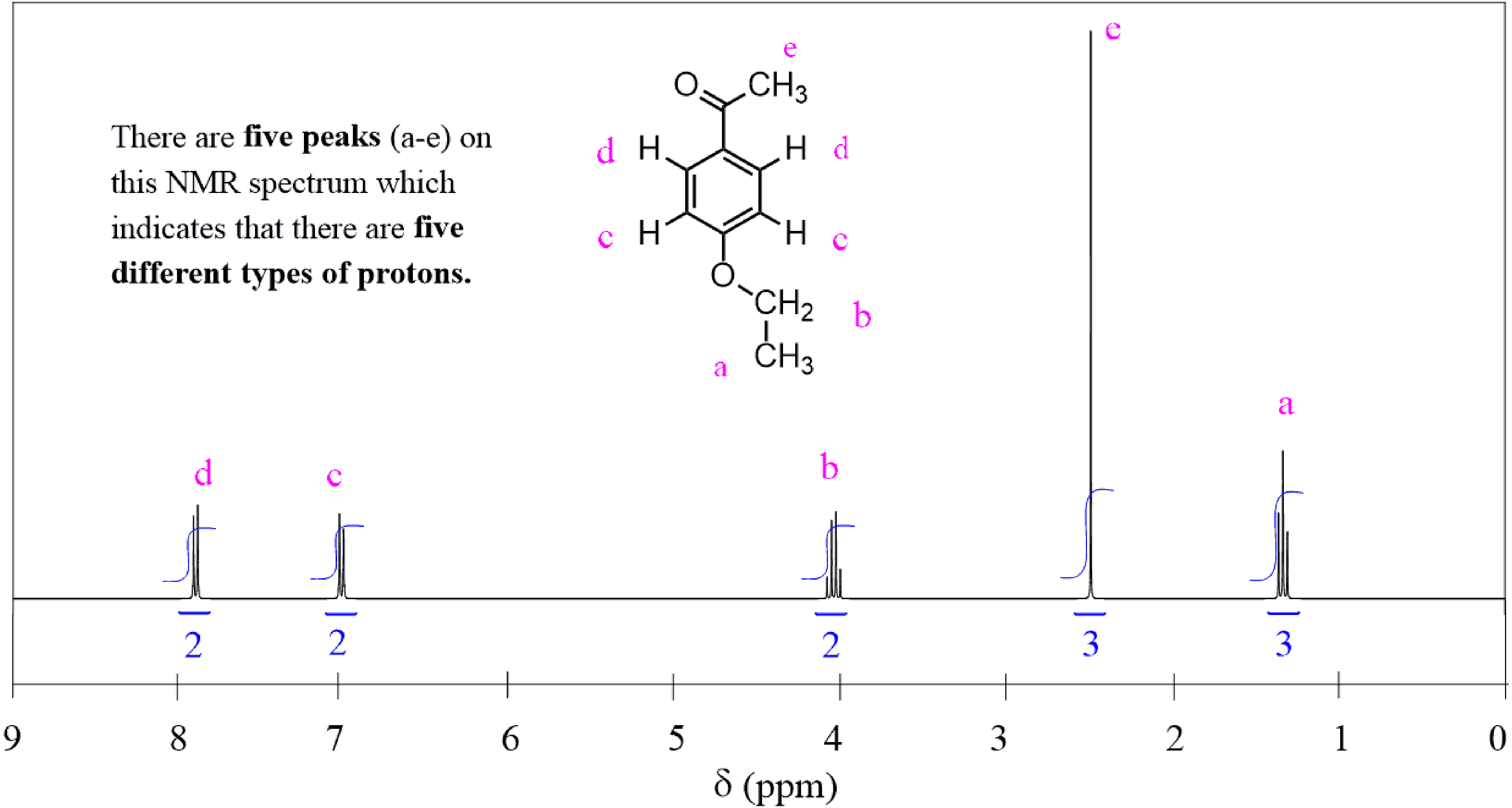


https://www.chemistrysteps.com/wp-content/uploads/2020/01/Integrals-NMR.png

NMR: Differences in neighbouring nuclei (1)

Principle: neighbouring nuclei influence each other's NMR energy levels, BUT this is also dependent on the chemical environment of each nucleus.

Example: ^{1}H NMR (for heteronuclear NMR, coupling cannot always be observed)



https://www.chemistrysteps.com/wp-content/uploads/2020/01/NMR-number-of-signals.png

NMR: Line splitting - intensities

Principle:

→ One signal splits in (n+1) lines, when coupling to n equivalent nuclei

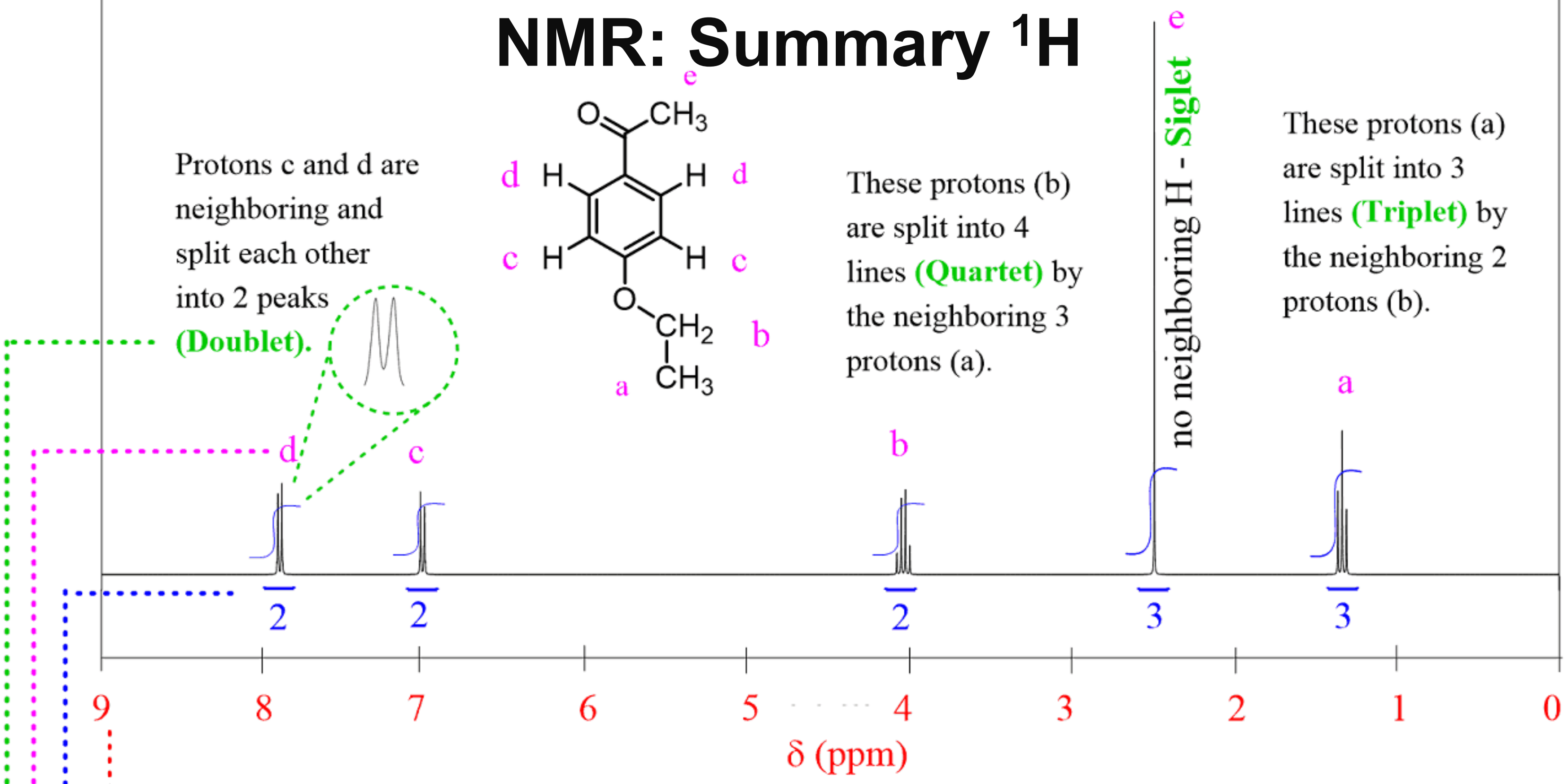
→ Integral of a resonance is proportional to number of neighbouring identical nuclei.

- Line intensities according to Pascal´s triangle

n		
0	1	Singlet
1	1 1	Doublet
2	1 2 1	Triplet
3	1 3 3 1	Quartet
4	1 4 6 4 1	Quintet
5	1 5 10 10 5 1	Sextet
6	1 6 15 20 15 6 1	Heptet

Fig. 4.22 Pascal's triangle. Coupling to n equivalent spin-$\frac{1}{2}$ nuclei produces $n+1$ lines, the relative intensities of which are given by the triangle.

NMR: Summary ^{1}H



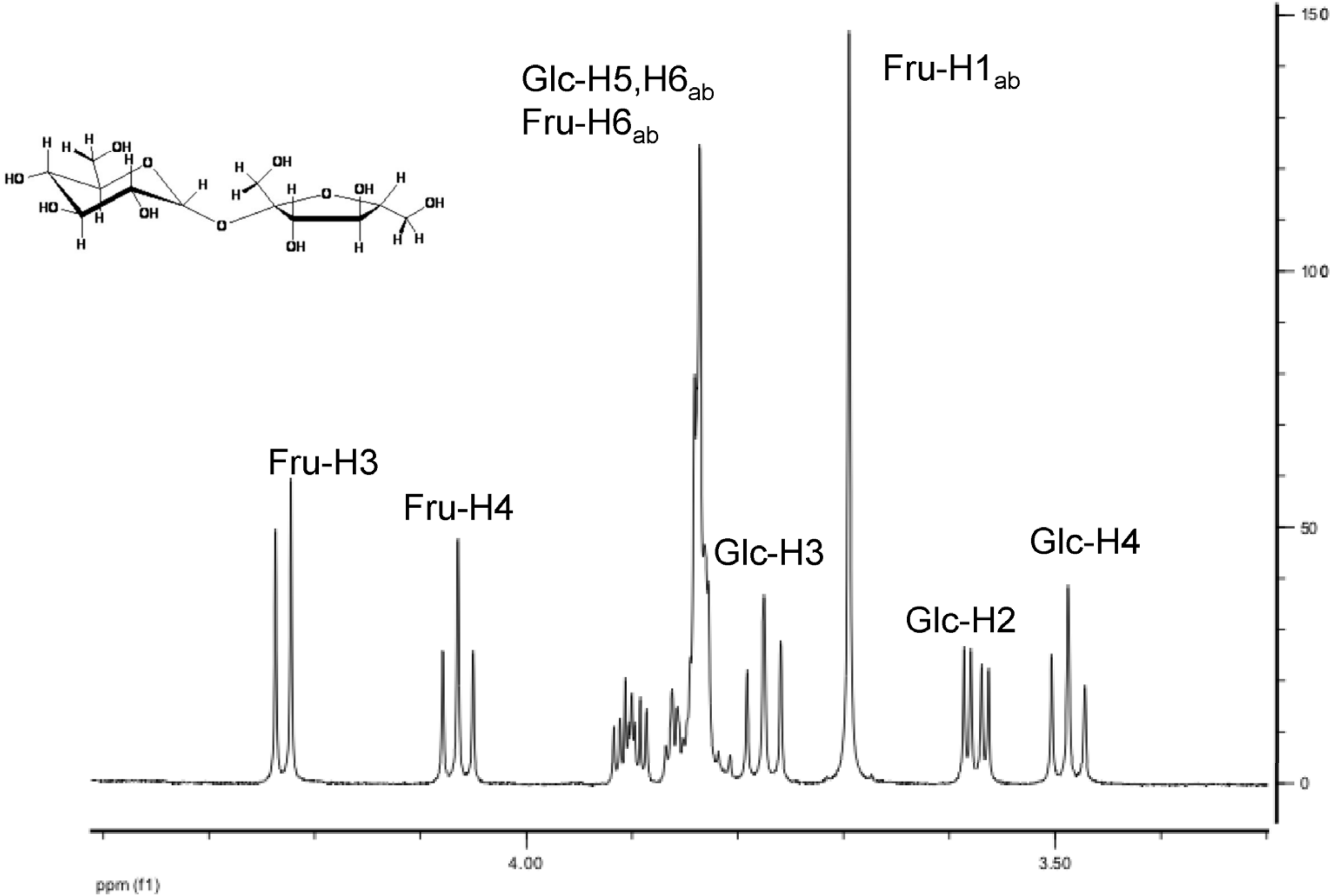
1. The **functional groups** that are present in the molecule. This is determined based on the **positions (ppm)** of the signals on the spectrum. Most often the scale goes from 0-12 ppm.

2. The **number of protons** represented by each signal. This measured by the **integration** which is the surface area under each signal peak(s).

3. The **number of different types of protons** in the molecule. This is determined by the **number of NMR signals**. Only non-equivalent protons give different signals. Chemically equivalent protons give one NMR signal regardless of their number.

4. The **spin-spin splitting** tells **how many protons are connected to the neighboring carbons**. This is determined by **the number of the peaks (signal multiplicity)** within the signal based on the **n+1 rule**, n being the number of neighboring protons.

^{1}H NMR Spectrum of Glucose



^{13}C-NMR

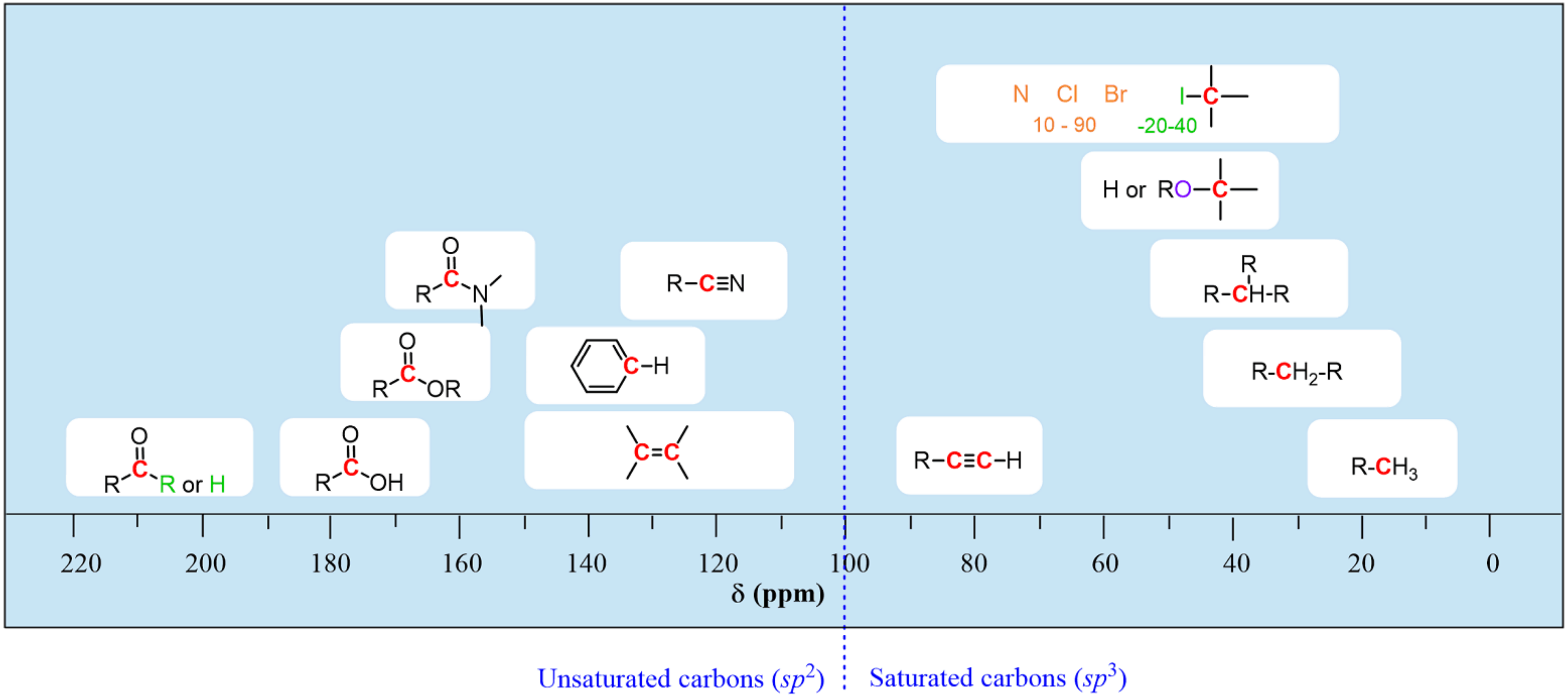
- Natural abundance of 1.1 % and low sensitivity
  → Acquisition takes more time

- No couplings observed
  - ^{13}C-^{13}C couplings because of low natural abundance
  - ^{1}H-^{13}C couplings because of decoupling during acquisition

- No integration because of decoupling

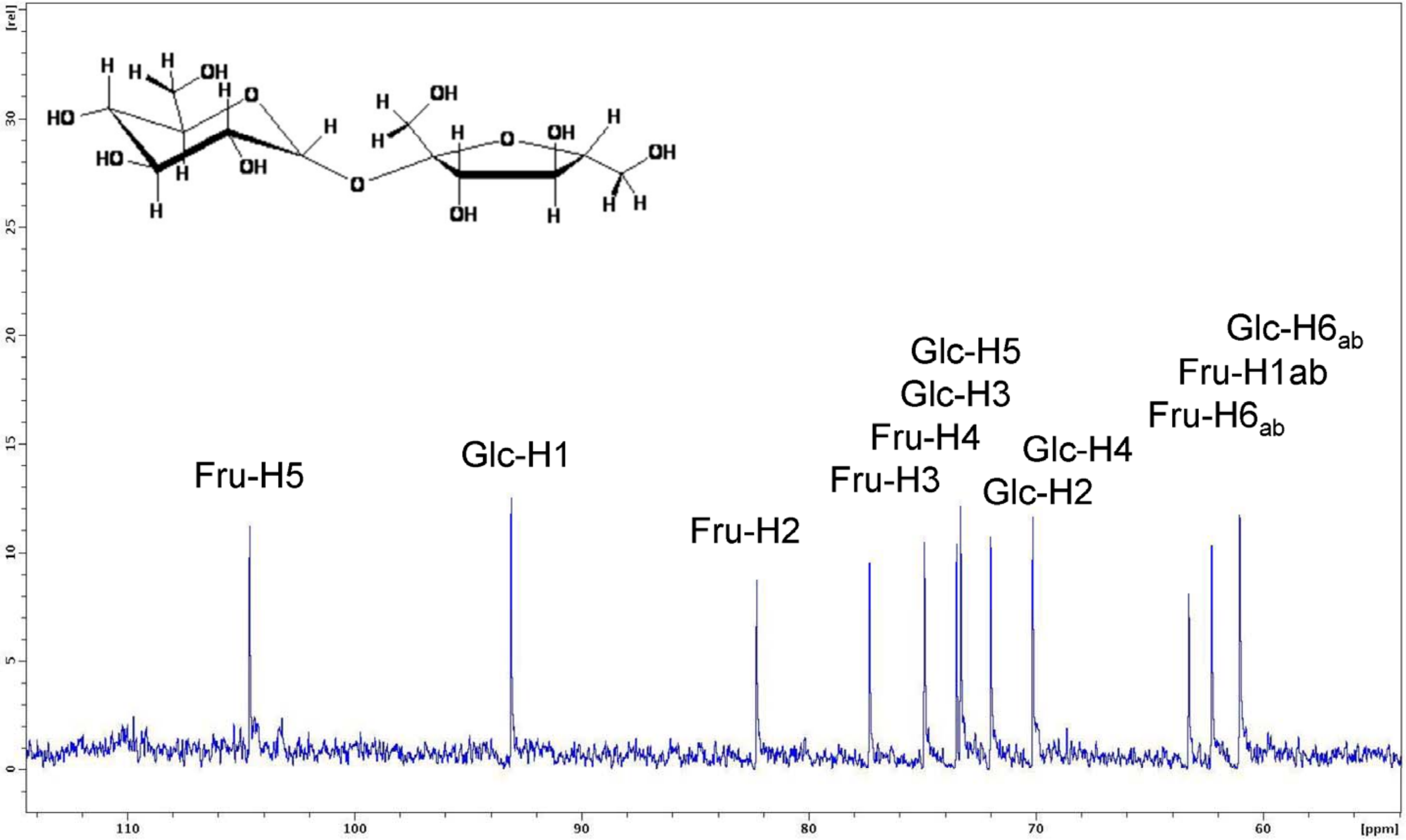
→ Determination of the number of different carbon atoms in a molecule

Chemical shift in ^{13}C NMR



https://www.chemistrysteps.com/wp-content/uploads/2020/02/13C-Carbon-NMR-Chemical-Shift-Values.png

^{13}C NMR Spectrum of Glucose



NMR Application for resolving protein structures (I)

NMR STUDIES OF STRUCTURE AND FUNCTION OF BIOLOGICAL MACROMOLECULES

Nobel Lecture, December 8, 2002

KURT WÜTHRICH

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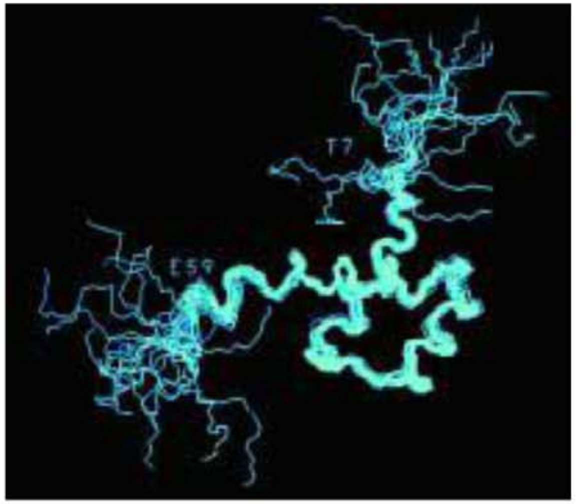
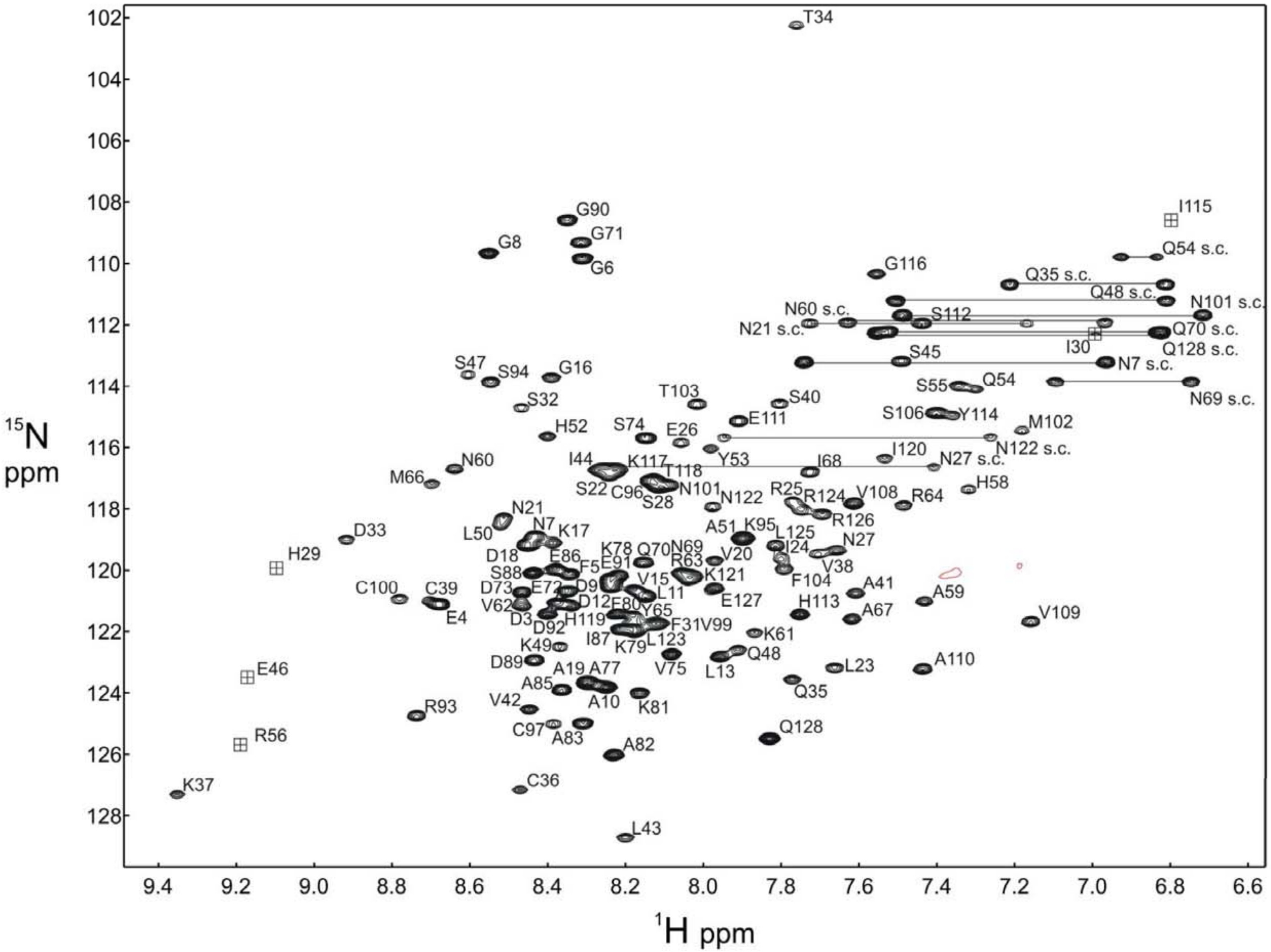


Figure 1. NMR structure of the *Antennapedia* homeodomain. A bundle of 20 superimposed conformers represents the polypeptide backbone. For the polypeptide segment 7–59 the tight fit of the bundle indicates that the structure is defined with high precision, whereas the two chain ends are disordered.

- Protein expression
  - Optimize expression yield
  - Optimize for high concentration, solubility, stability
  - Express ^{15}N- and ^{15}N-/ ^{13}C-labelled protein

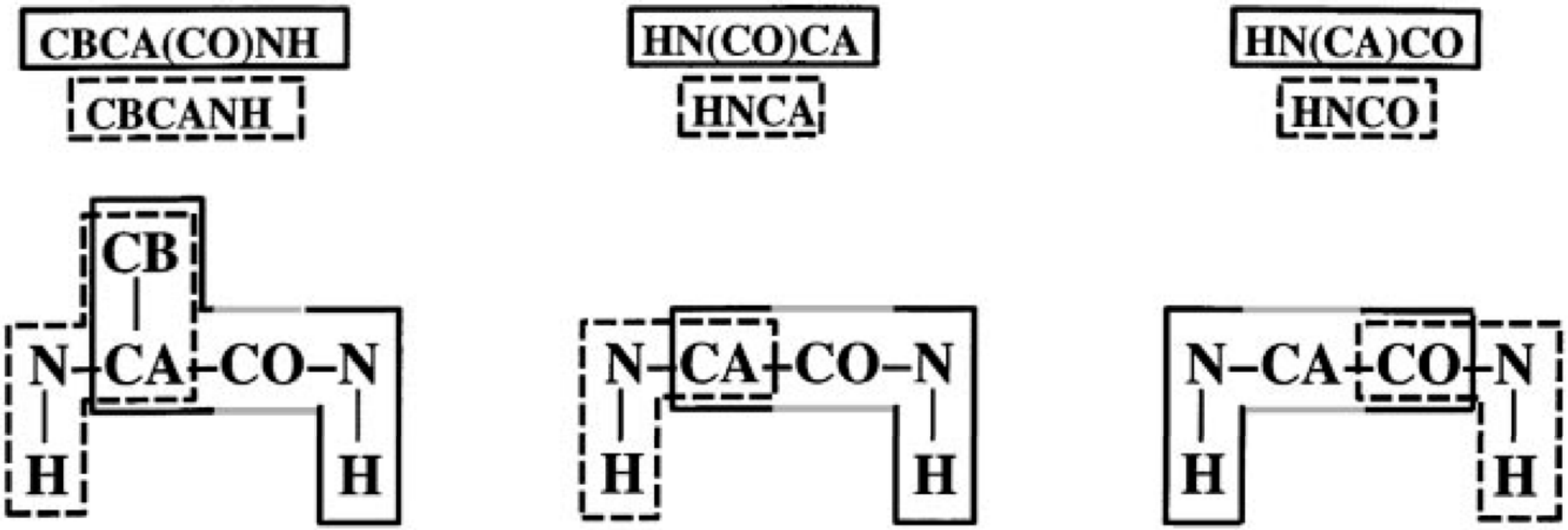
NMR Application for resolving protein structures (II)

→ combination of ^1H and ^{13}C NMR ("2-dimensional NMR")



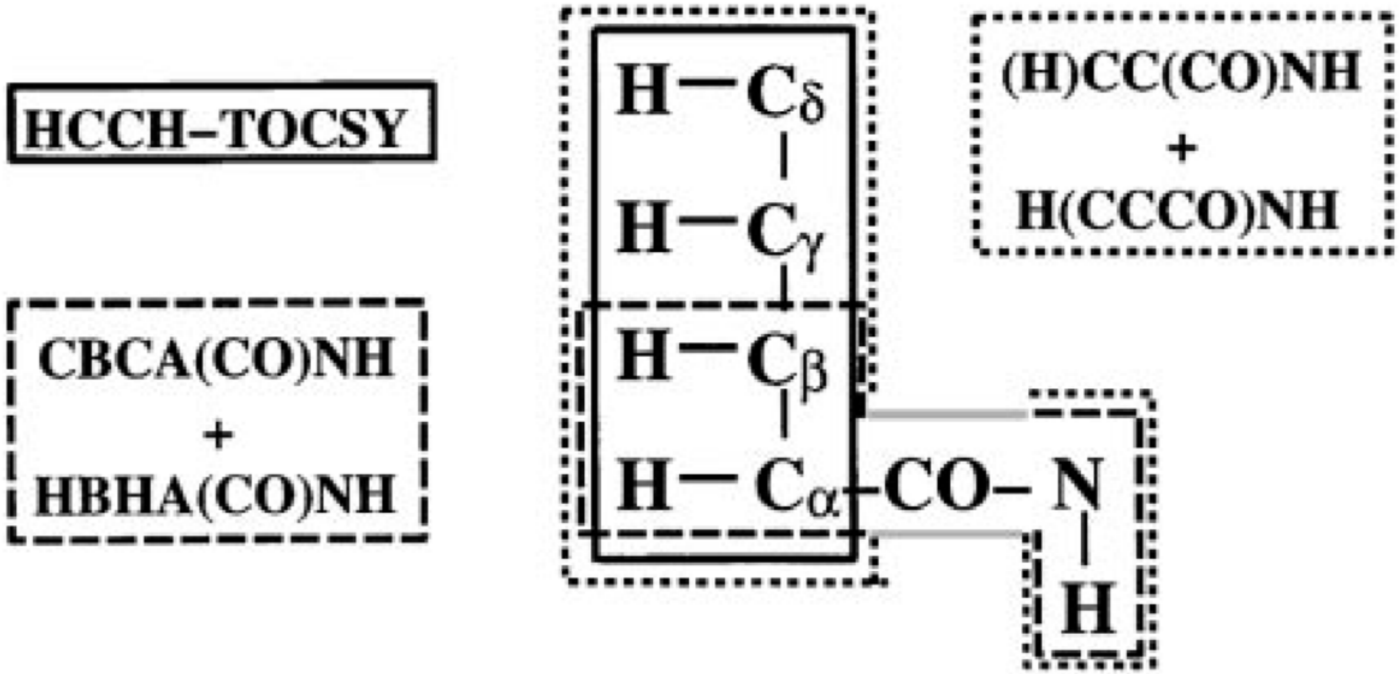
NMR Application for resolving protein structures (III) → Structure assignment (1)

- Sequential Assignment: identify amino acid spin systems and their sequence position



NMR Application for resolving protein structures (IV)
→ Structure assignment (2)

- Side chain assignment: determine all relevant chemical shifts of ^{1}H, ^{13}C and ^{15}N



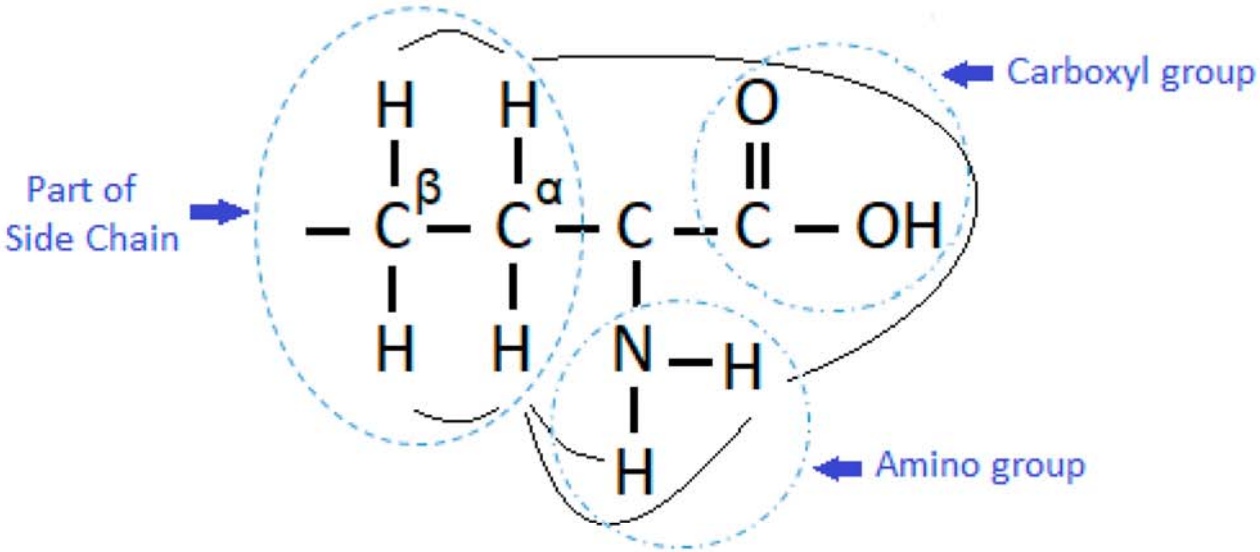
NMR Application for resolving protein structures (IV) → Structure assignment (3)

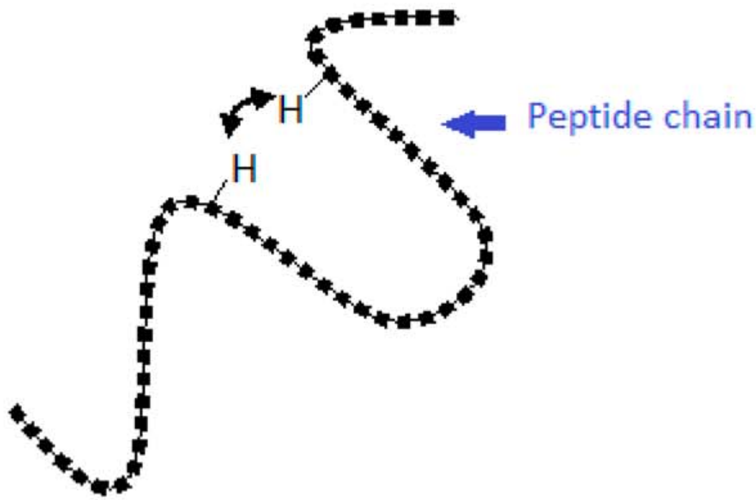
Use of the Nuclear Overhauser Effect (NOE)

→ In this application, the NOE differs from the application of spin-spin coupling in that the NOE occurs through space, not through chemical bonds. → **Modification of NMR line intensities**

→ Thus, atoms that are in close proximity to each other can give a NOE, whereas spin coupling is observed only when the atoms are connected by 2–3 chemical bonds.

→ The inter-atomic distances derived from the observed NOE can often help to confirm a precise molecular conformation, i.e. the three-dimensional structure of a molecule.





Through bound (scalar) coupling (a); through space coupling—nuclear Overhauser effect (NOE) (b).

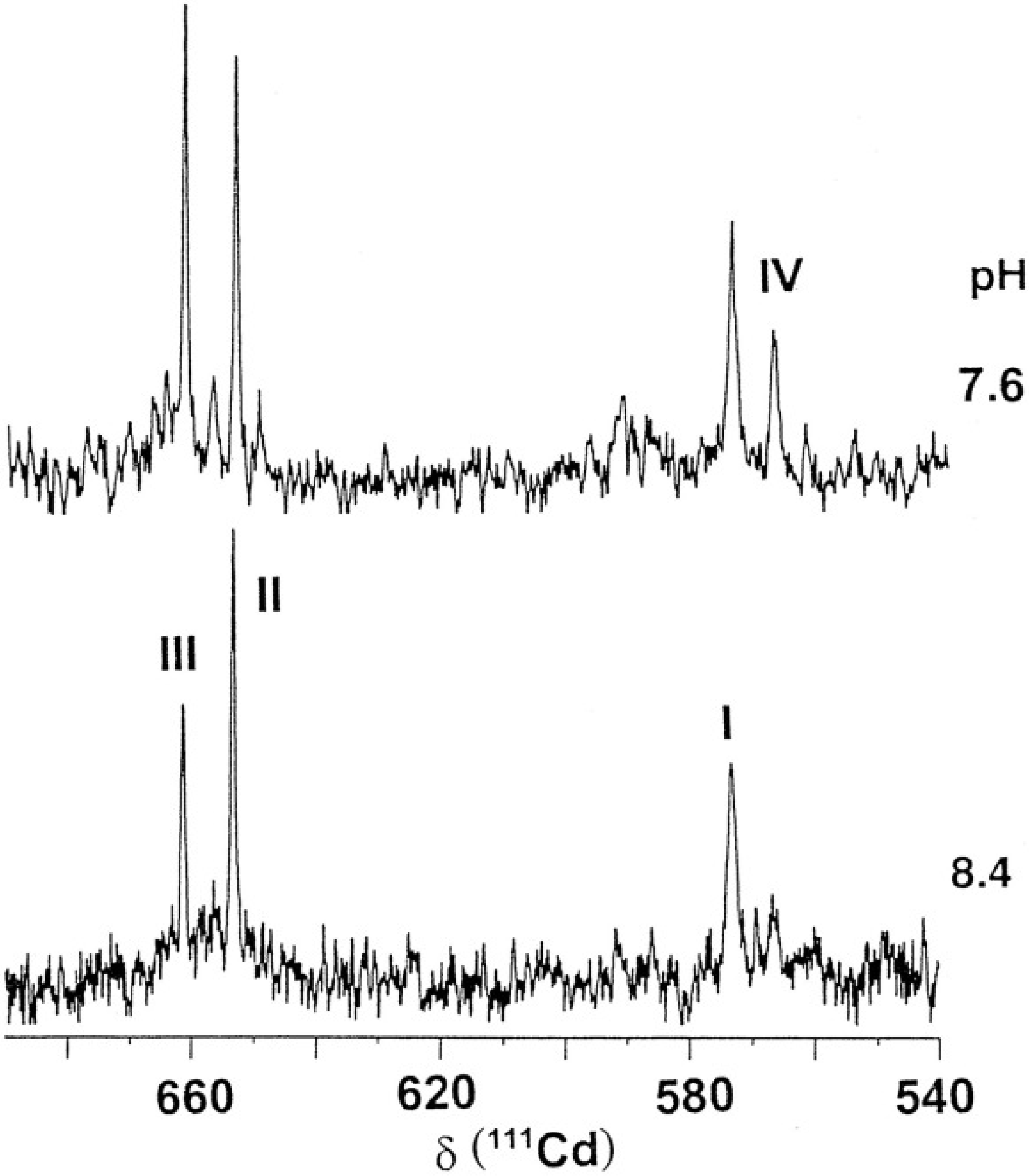
Markoska T, Vasiljevic T, Huppertz T. Unravelling Conformational Aspects of Milk Protein Structure—Contributions from Nuclear Magnetic Resonance Studies. Foods. 2020; 9(8):1128. https://doi.org/10.3390/foods9081128

^{111}Cd NMR Example: Coordination of Cd^{2+} by Prokaryotic Metallothionein

Use of chemical shift:

→ Two peaks, II and III (654 and 661 ppm), are in the chemical shift range expected for CdS4 coordination, and peak I (572 ppm) is in the range for CdNS3 or CdN2S2 coordination.

→ At pH 7.6 an additional peak (IV) is apparent at 567 ppm.



Michael J. Daniels, Jennifer S. Turner-Cavet, Richard Selkirk, Hongzhe Sun, John A. Parkinson, Peter J. Sadler, Nigel J. Robinson
Journal of Biological Chemistry 273, 22957-22961 (September 1998) DOI: 10.1074/jbc.273.36.22957

All slides of my lectures can be downloaded from my workgroup homepage

Biology Centre CAS → Institute of Plant Molecular Biology → Departments → Department of Plant Biophysics and Biochemistry,

or directly

http://webserver.umbr.cas.cz/~kupper/AG_Kuepper_Homepage.html