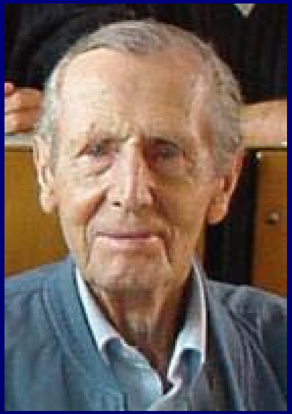
Magnetic Resonance Spectroscopy (EPR, NMR) as structural tools

Hendrik Küpper, Advanced Course on Bioinorganic Chemistry & Biophysics of Plants, summer semester 2026

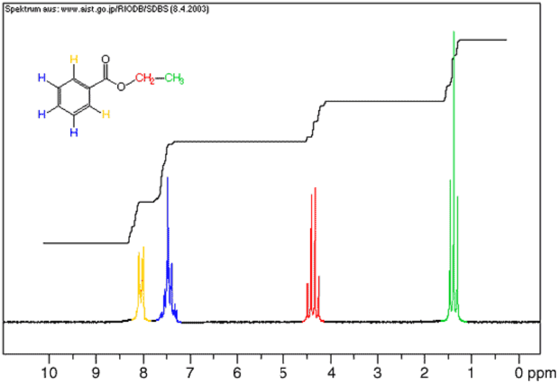
based on a lecture of Peter Kroneck, Universität Konstanz

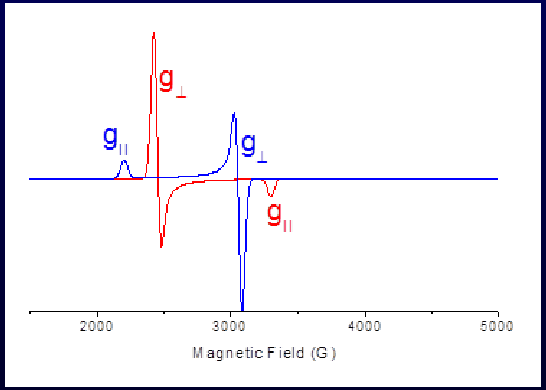
Introduction: EPR and NMR

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



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



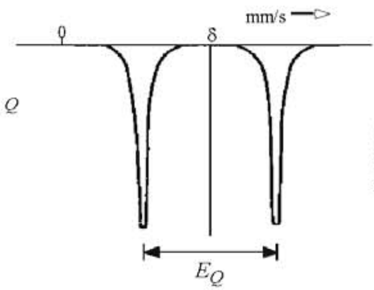


→ Electron Paramagnetic Resonance and Nuclear Magnetic Resonance are non-invasive techniques. They can be applied to living systems *in vivo* to obtain images, such as distribution of H_2O, O_2, or NO in tissues, or for analysis of molecular structures, also of metal complexes

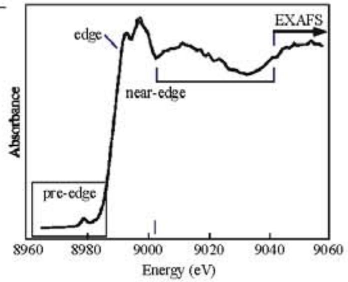
Spectroscopic Techniques - Energy

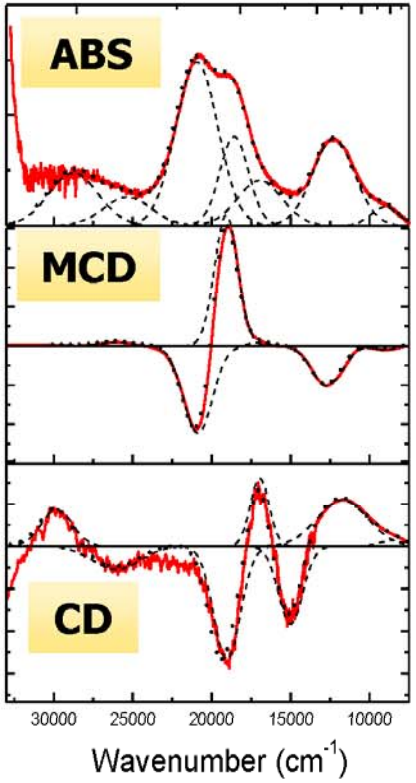
	Gamma	X-Ray	UV/Vis	Infrared	Microwave	Radiowave
eV	14000	8000 2000	4 - 1	0.1-0.01	10^{-4} -10^{-5}	10^{-6} -10^{-7}

Möss-bauer

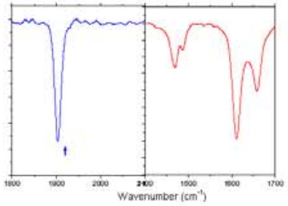


XAS EXAFS

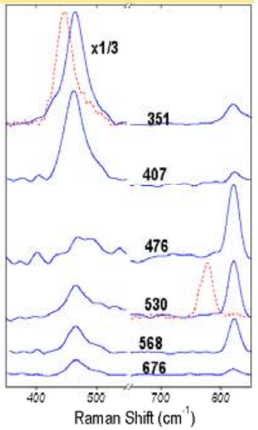




IR

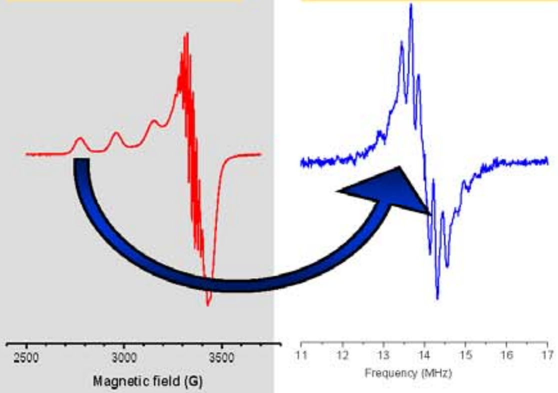


RAMAN



EPR

ENDOR



NMR

EPR + NMR =
Electron Nuclear
Double Resonance

Circular Dichroism
Magnetic Circular Dichroism

Lecture slide of Peter Kroneck

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, $S \neq 0$

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 ($Tyr^{\bullet}$,$Trp^{\bullet}$,$Gly^{\bullet}$,$Cys^{\bullet}$)**
3. **Radical states of cofactors (Semiquinones, Flavins ...)**
4. **Inorganic radicals ($NO^{\bullet}$, O_2 , $O_2^{\bullet -}$, $HO^{\bullet}$....)**
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**

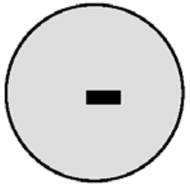
Lecture slide of Peter Kroneck

Basic Properties of Electrons

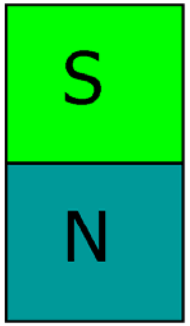
An Electron has the following properties:

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

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

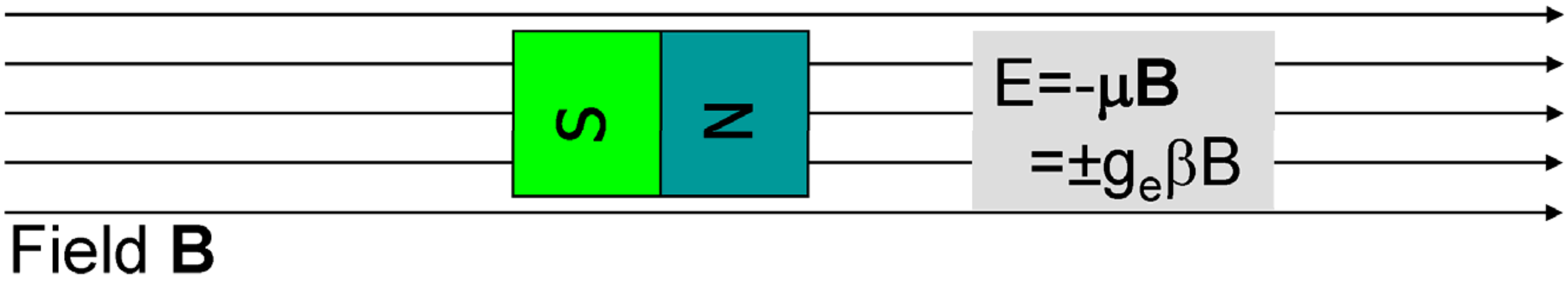


Electric monopole



Magnetic Dipole

The Magnetic Dipole in a Magnetic Field:



$$E = -\mu B$$
$$= \pm g_e \beta B$$

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:

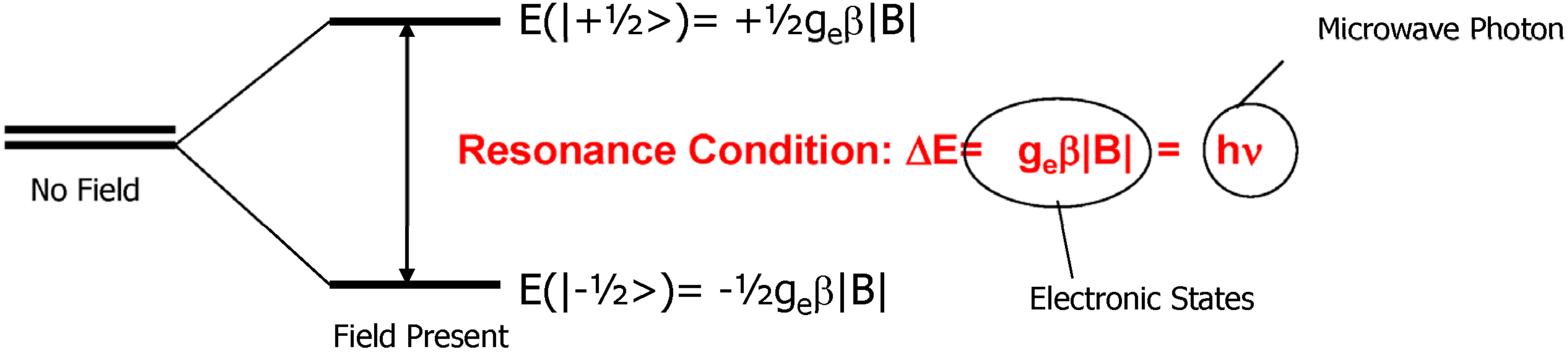
|+1/2> and |-1/2>

„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.



$E(|+\tfrac{1}{2}>) = +\tfrac{1}{2} g_e \beta |B|$

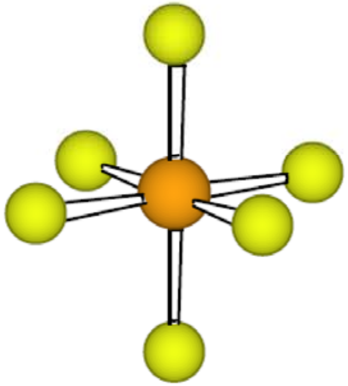
Resonance Condition: $\Delta E = g_e \beta |B| = h\nu$

$E(|-\tfrac{1}{2}>) = -\tfrac{1}{2} g_e \beta |B|$

Lecture slide of Peter Kroneck

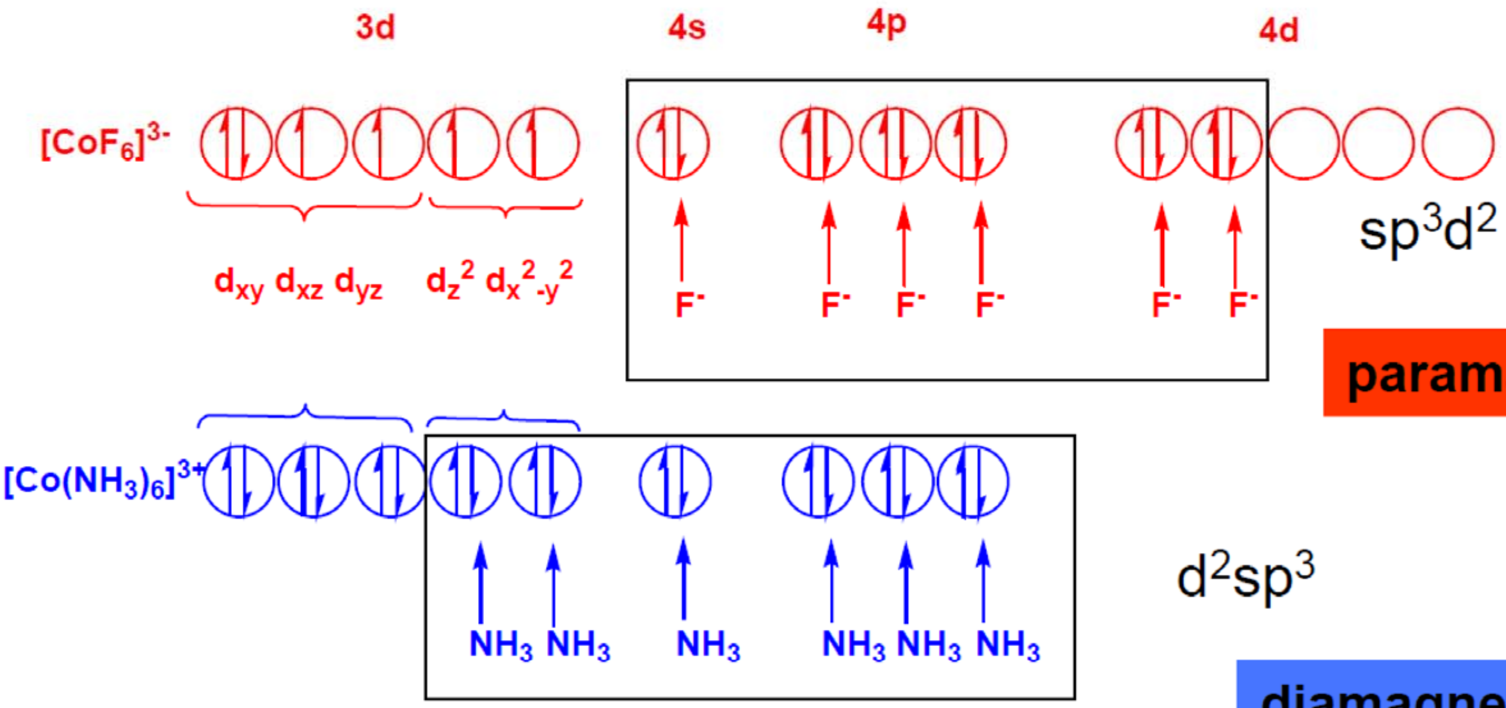
Remember: Valence Bond Theory

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

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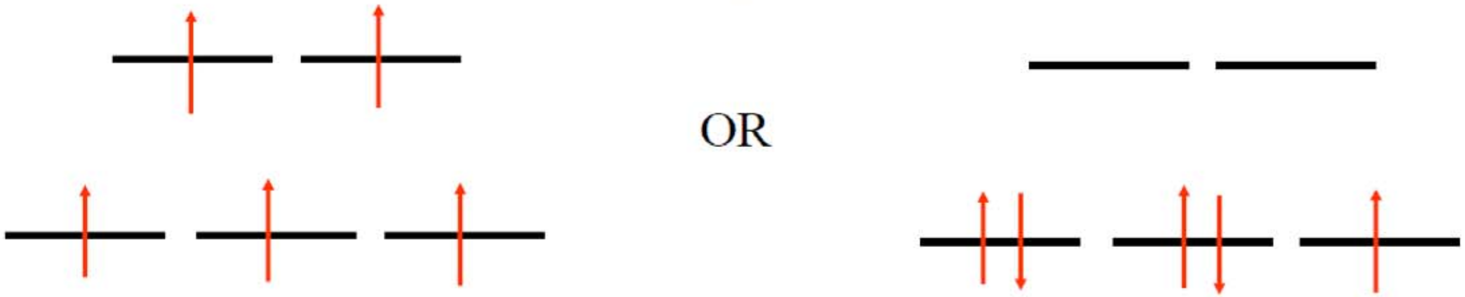
Color and Magnetism

Variable Spin States of Metal Centers

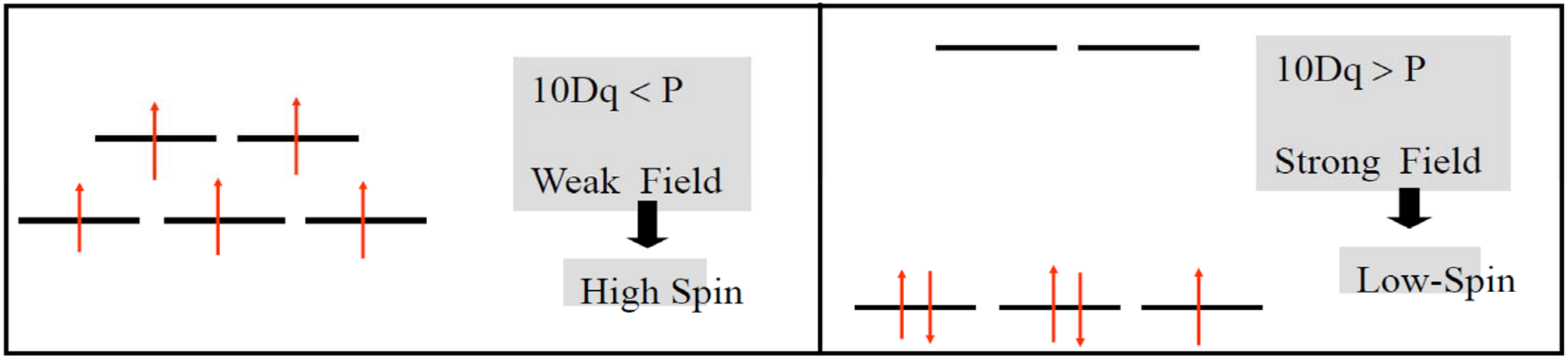
For a d^5 configuration, Fe(III)



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

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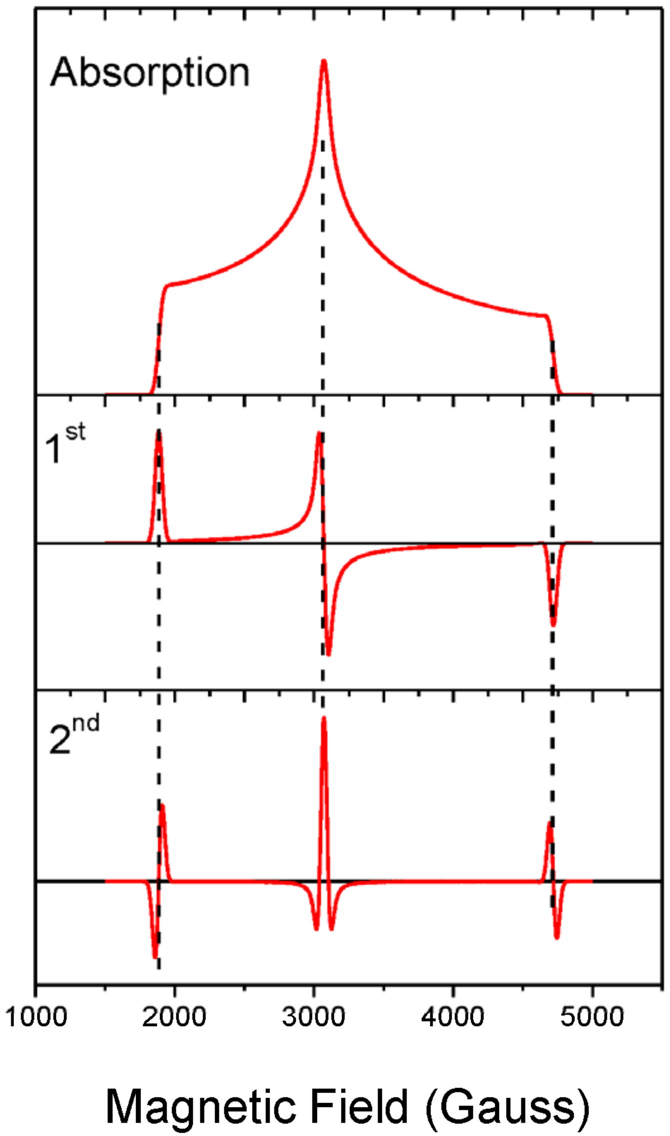
Presentation of EPR Spectra



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

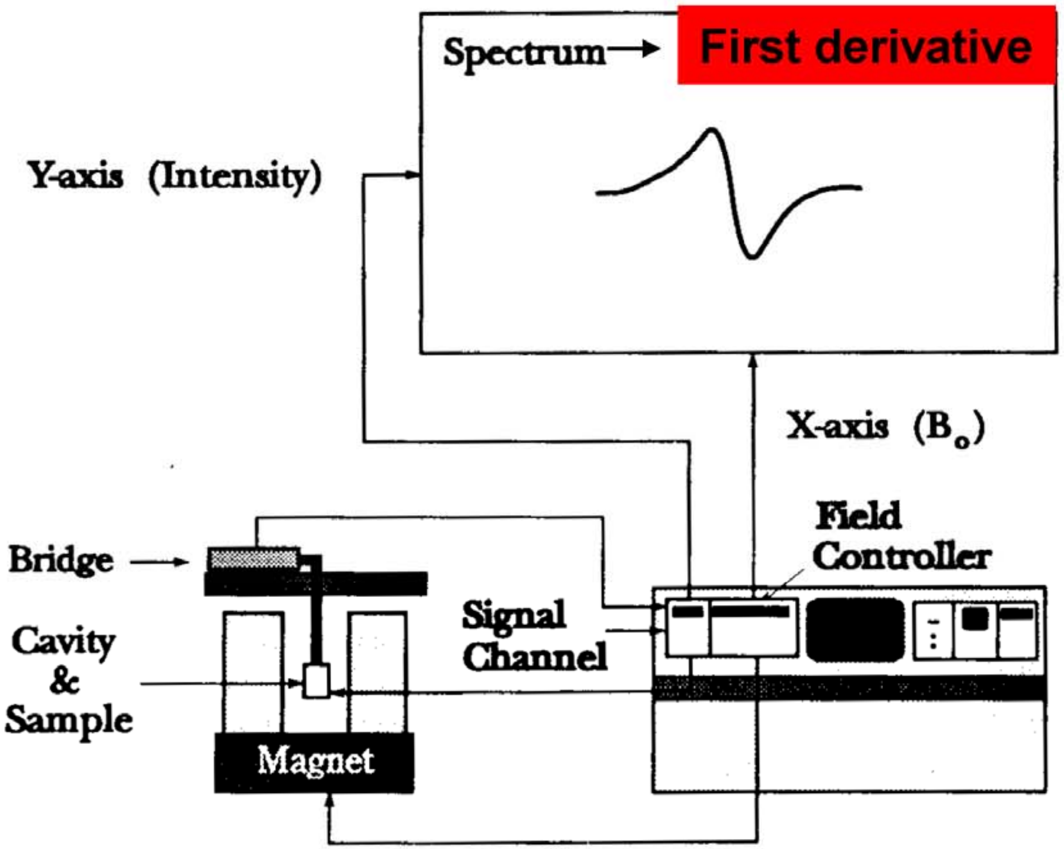
1T = 10 000 G
1 mT = 10 G

Typical resonance field

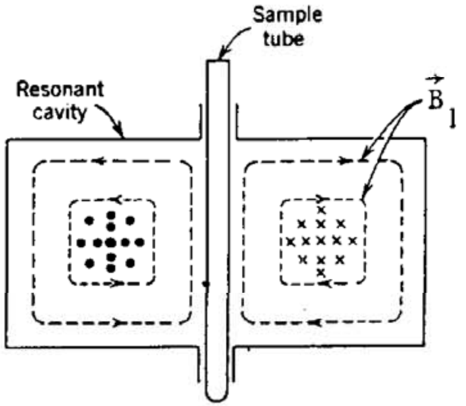
$$B_{res} \sim 3000\ \mathrm{G} = 0.3\mathrm{T}$$

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

Multifrequency EPR

In EPR we usually FIX the microwave frequency v (because of the cavity) and VARY the magnetic field B.

The magnetic field scale is inversely proportional to energy !

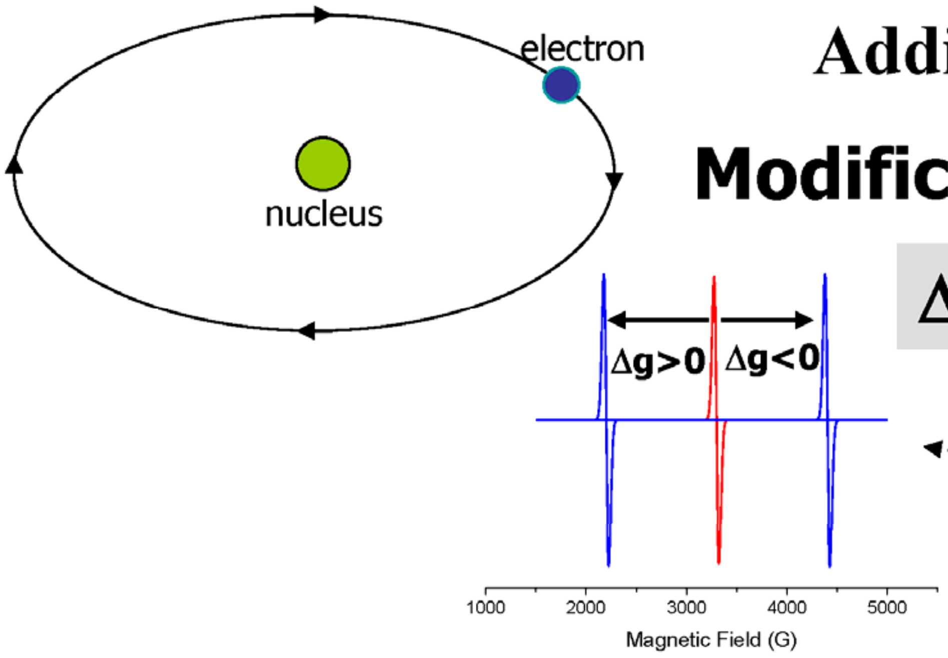
Thus, for every frequency we need a different Cavity, and we might have to change the magnet:

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

Lecture slide of Peter Kroneck

$g_{effective}$ /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

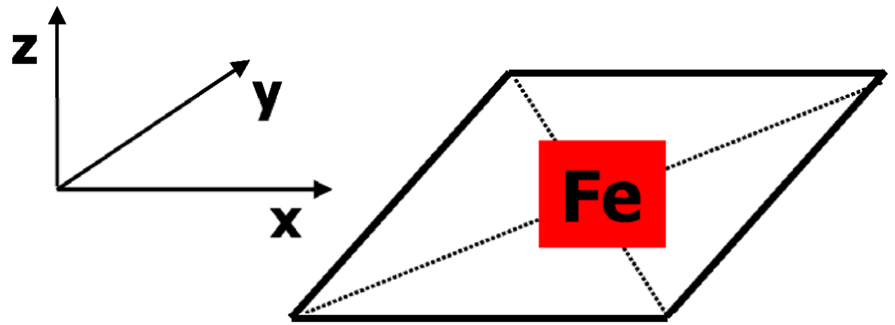
Modification of Resonance Condition:

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

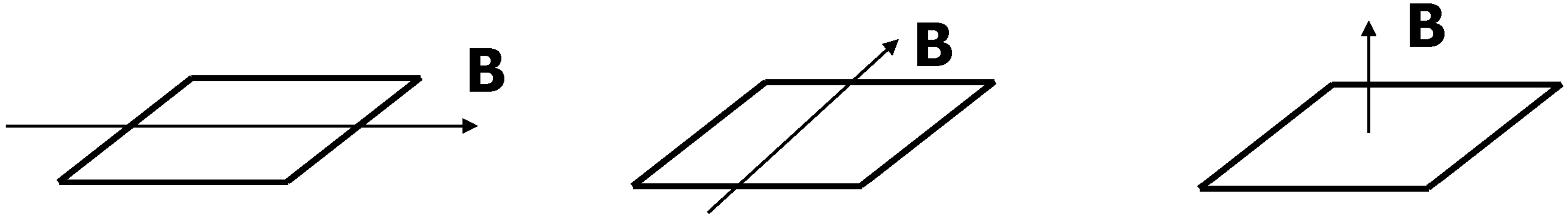
MOLECULAR Quantity=$g_e+\Delta g$

Anisotropy of g

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



Consider three extreme cases:



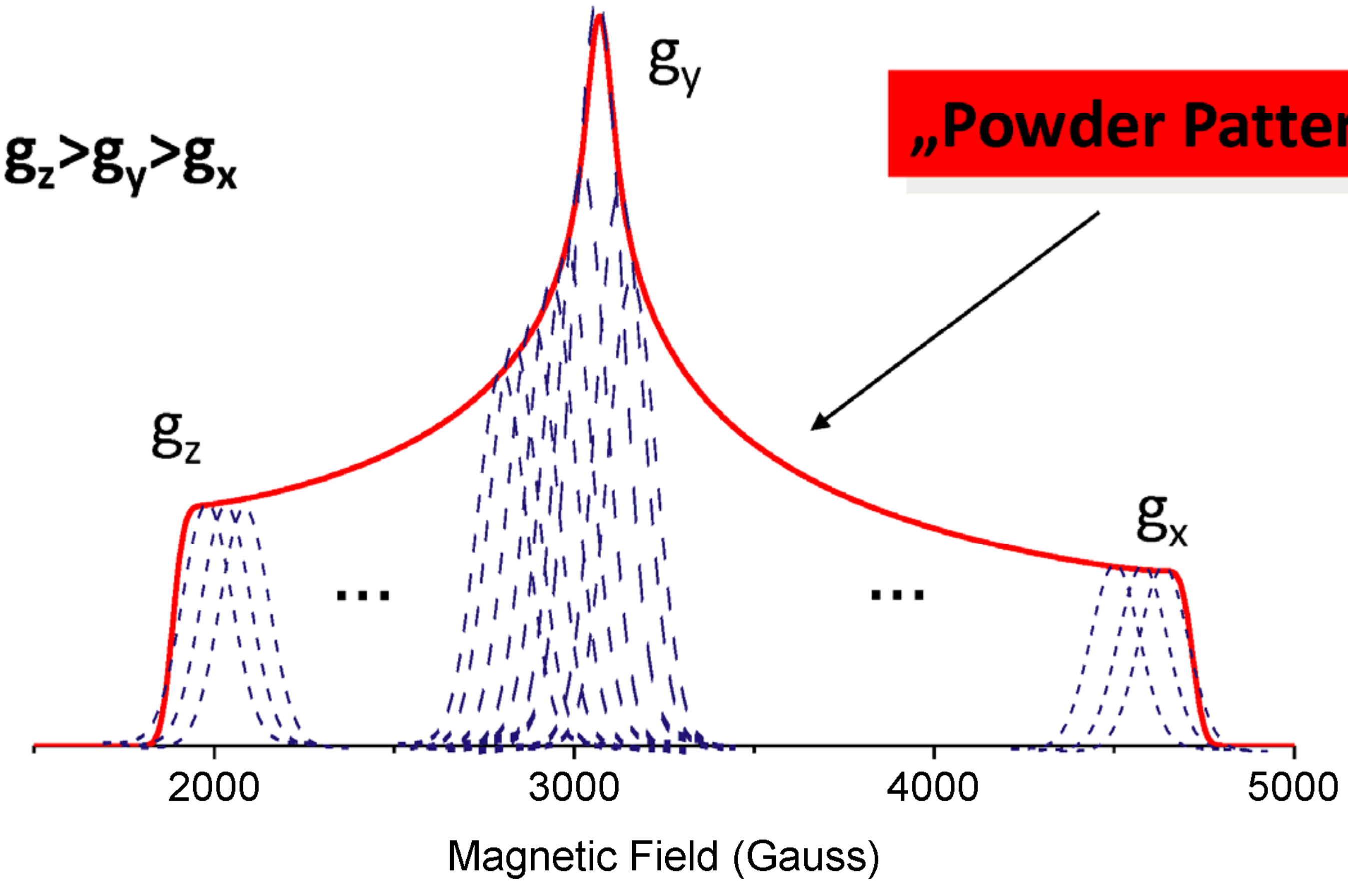
$h\nu=-B_x\mu_x=\beta B_x\mathbf{g_x}$ $h\nu=-B_y\mu_y=\beta B_y\mathbf{g_y}$ $h\nu=-B_z\mu_z=\beta B_z\mathbf{g_z}$

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 $g_z > g_y > g_x$

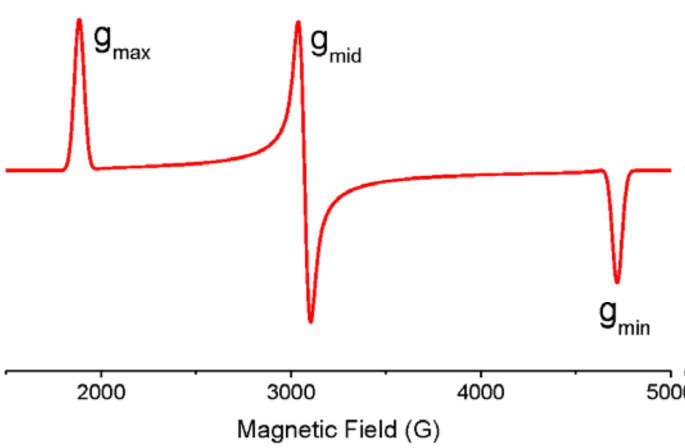


„Powder Pattern“

„Dialect“ for Powder Patterns

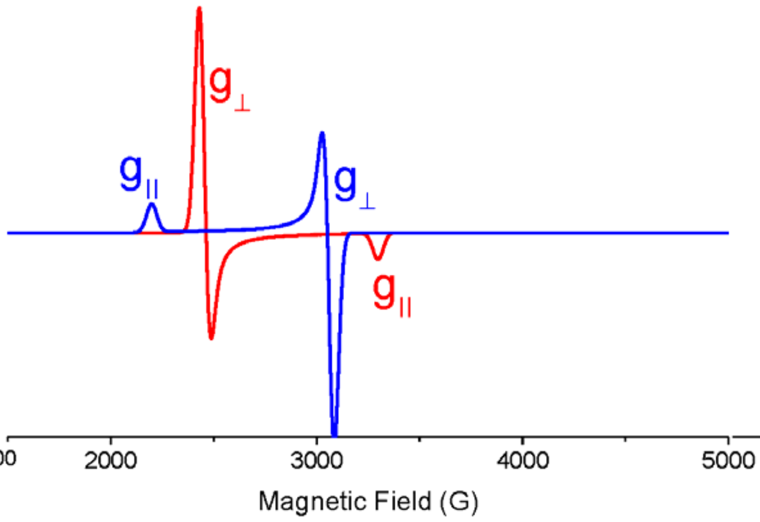
RHOMBIC

$g_x \neq g_y \neq g_z$



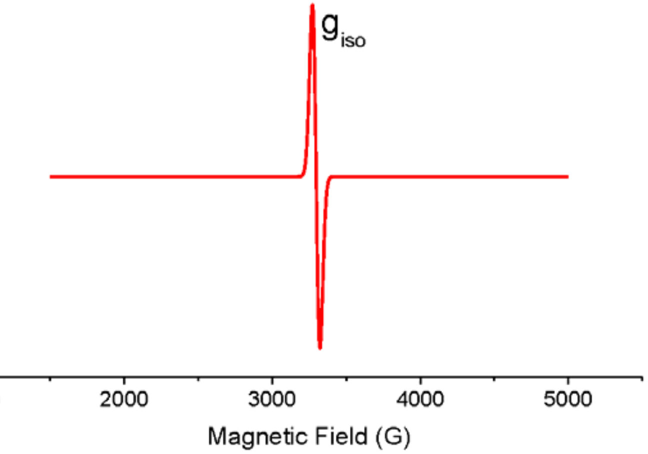
AXIAL

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



ISOTROPIC

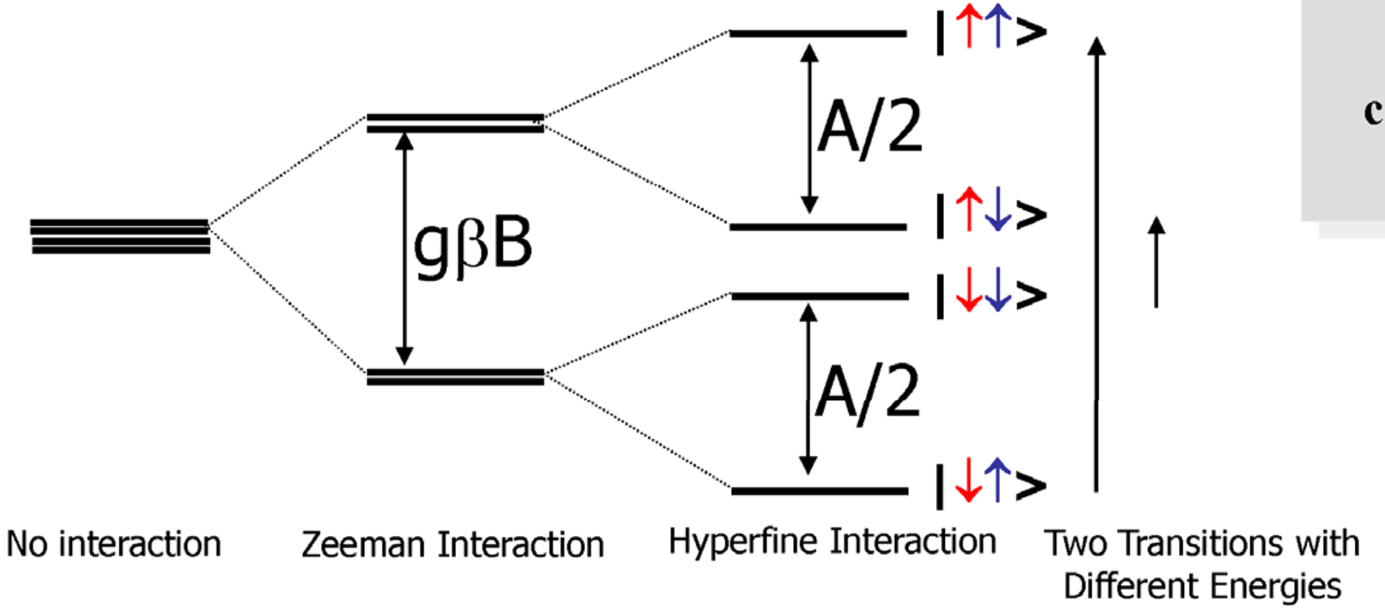
$g_x = g_y = g_z$



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



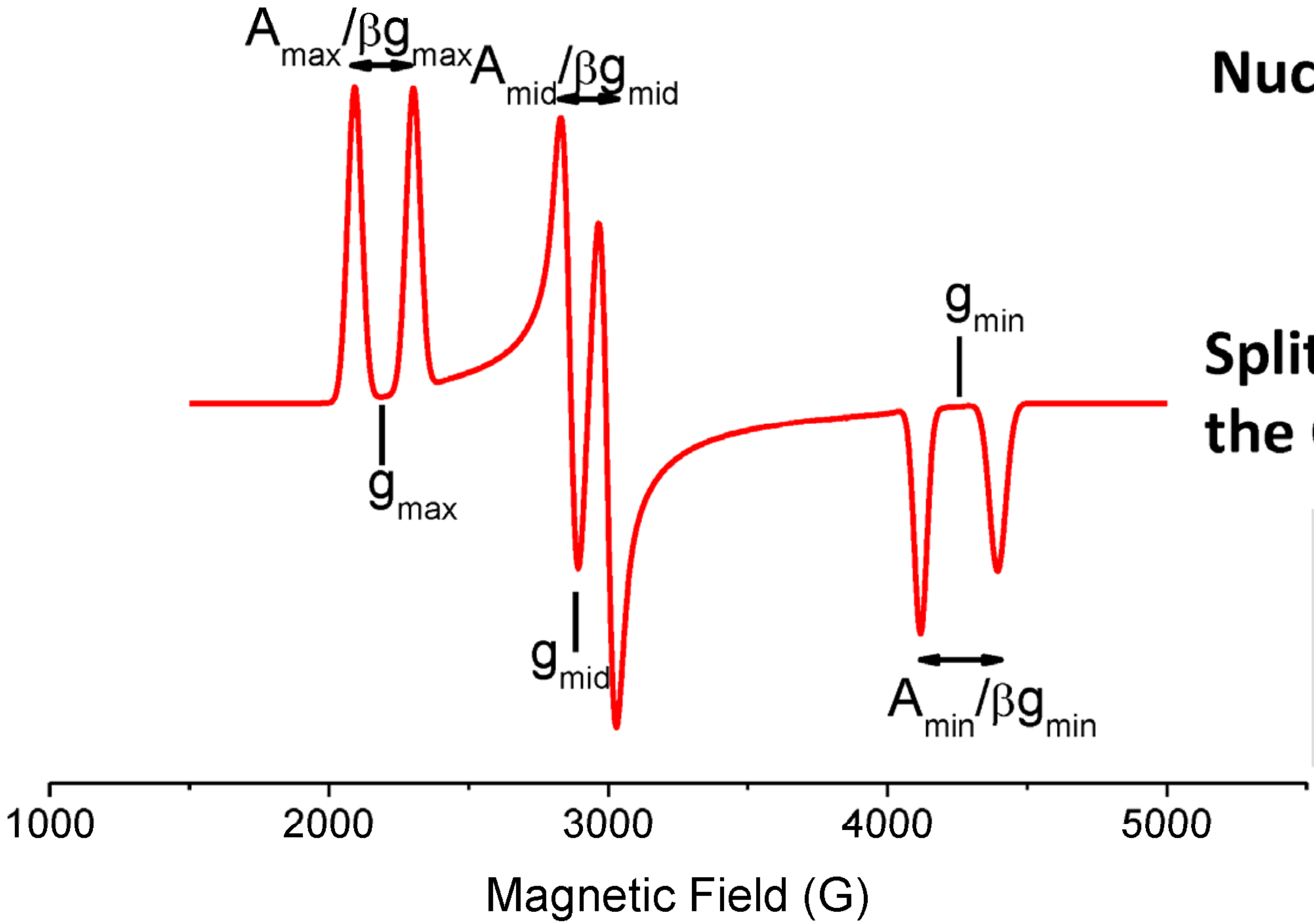
Selection Rule:
The Nuclear Spin does not change in an EPR Transition
$\Delta m_S = 1; \Delta m_I = 0$

↑ Electron Spin

↑ Nuclear Spin

Lecture slide of Peter Kroneck

EPR Spectrum with Hyperfine Structure



Nuclear Spin I = 1/2

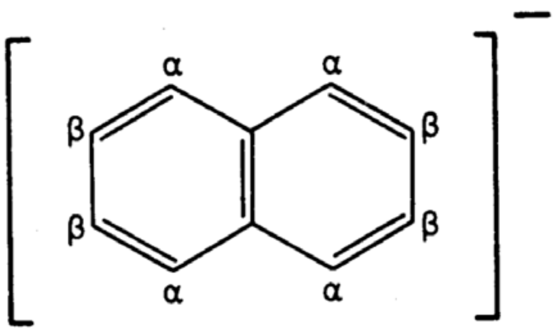
2I+1 Lines

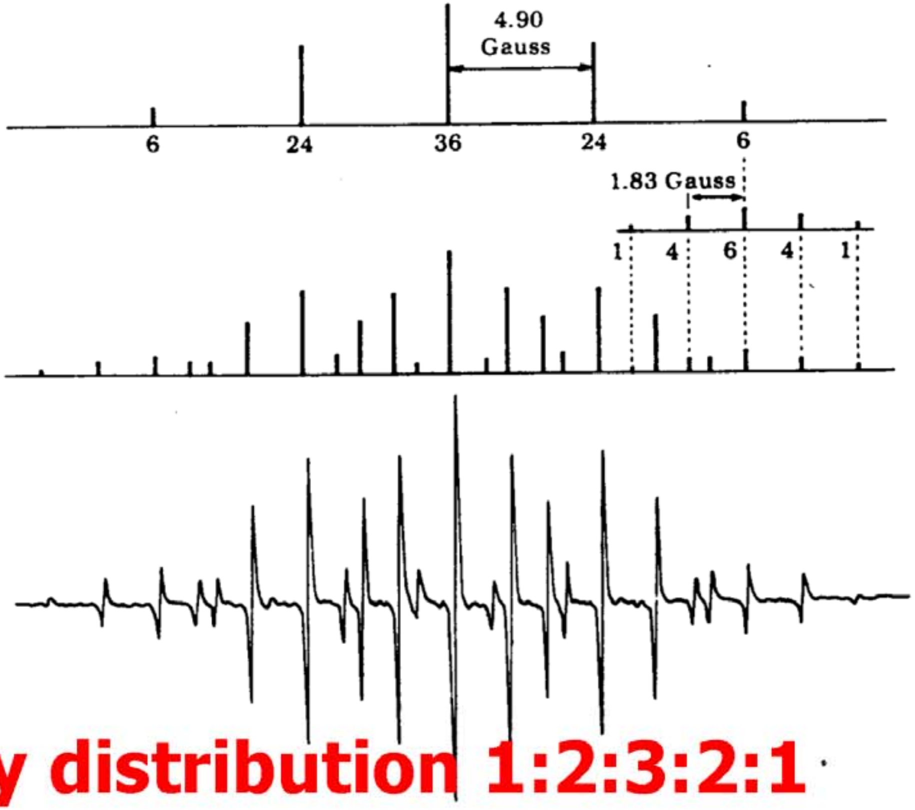
Splitting Depends on the Orientation

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

Lecture slide of Peter Kroneck

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

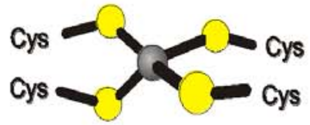
a_α=4.9 G

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

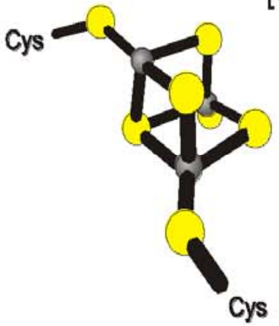
Rubredoxin



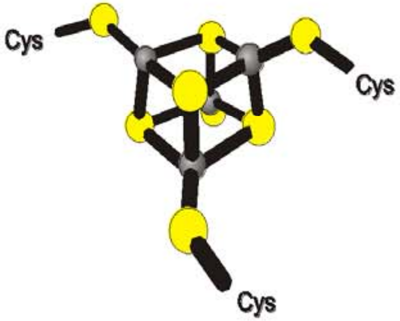
[2Fe-2S] Ferredoxin

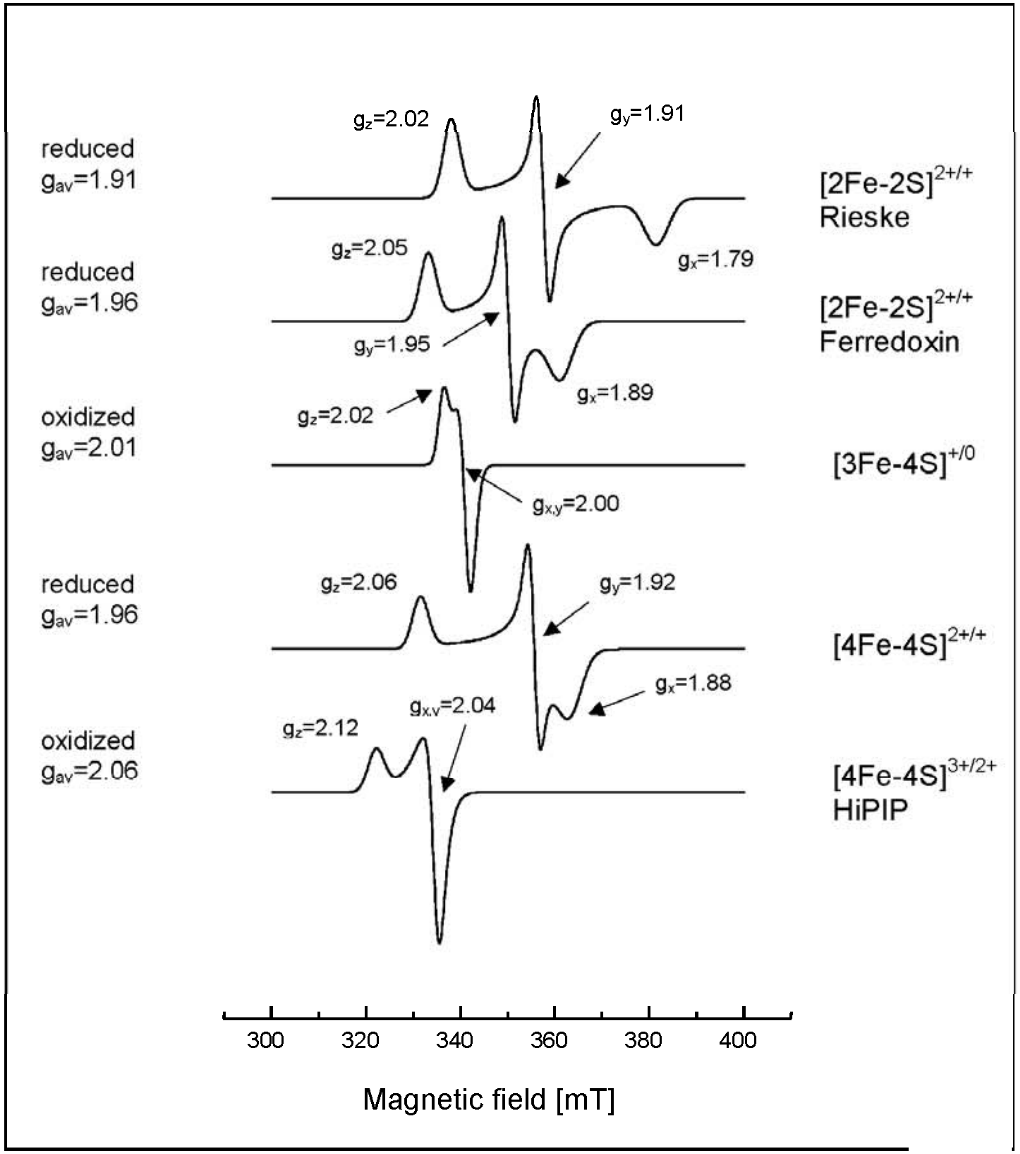
[2Fe-2S] Rieske center

[3Fe-4S]

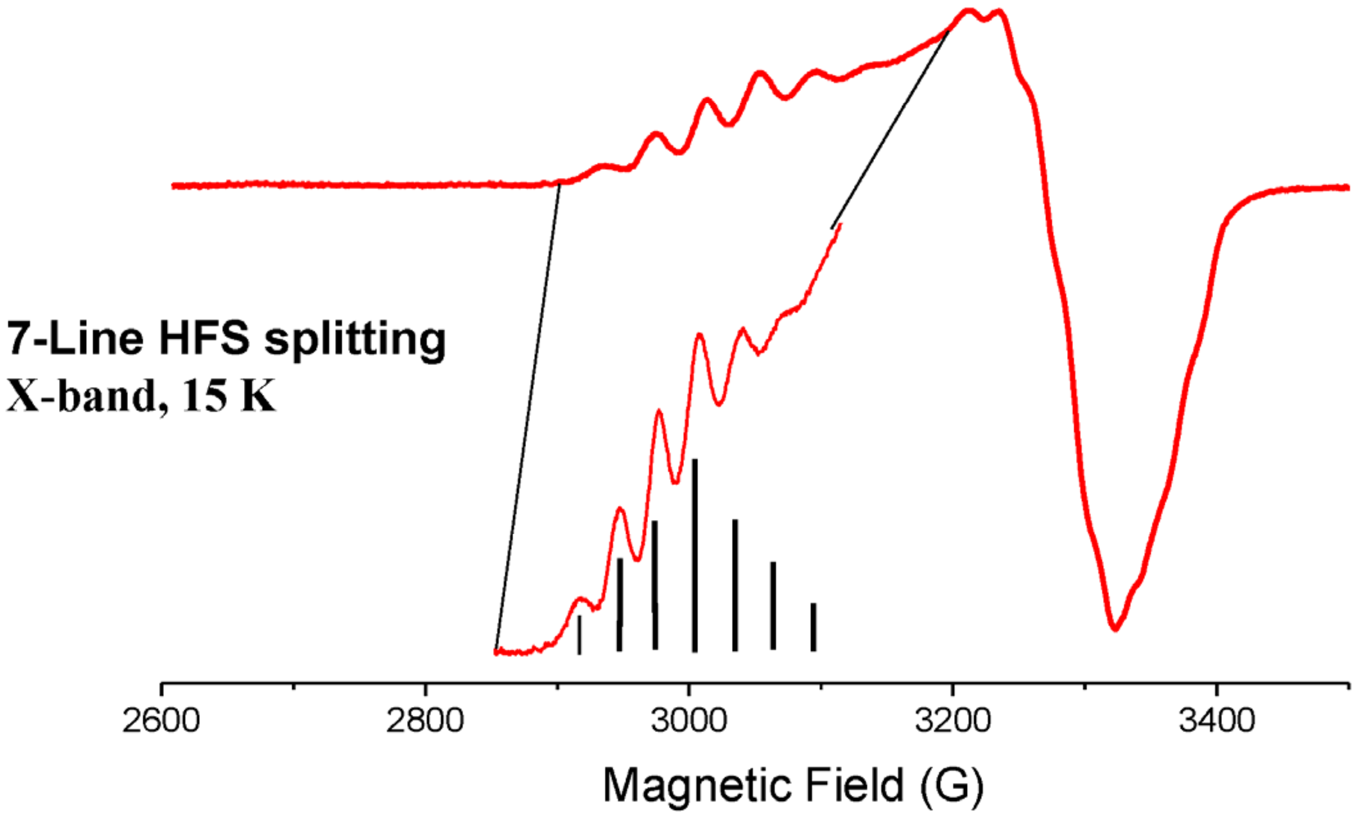


[4Fe-4S]



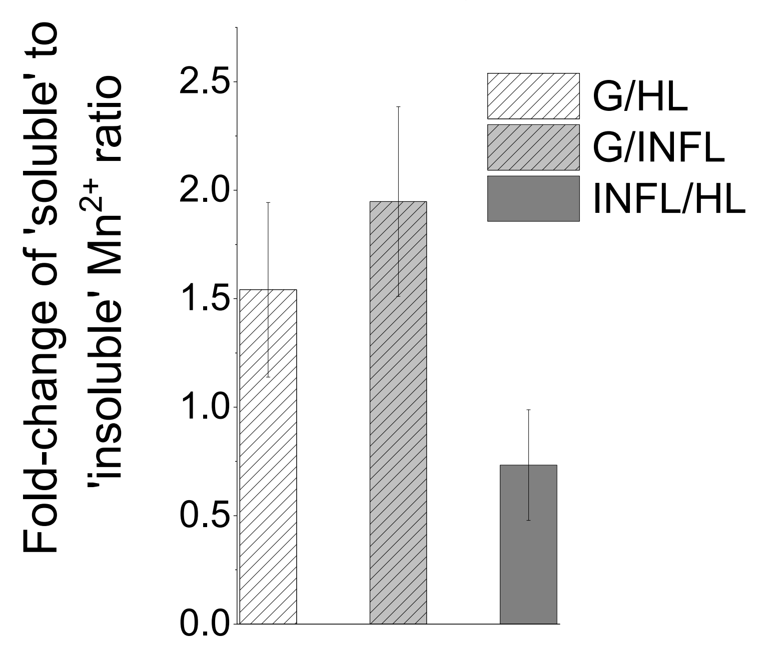


Coyle CL, Zumft WG, Kroneck PMH, Körner H, Jakob W (1985)
Purple Nitrous Oxide Reductase. Eur. J. Biochem., **153**, 459-467



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

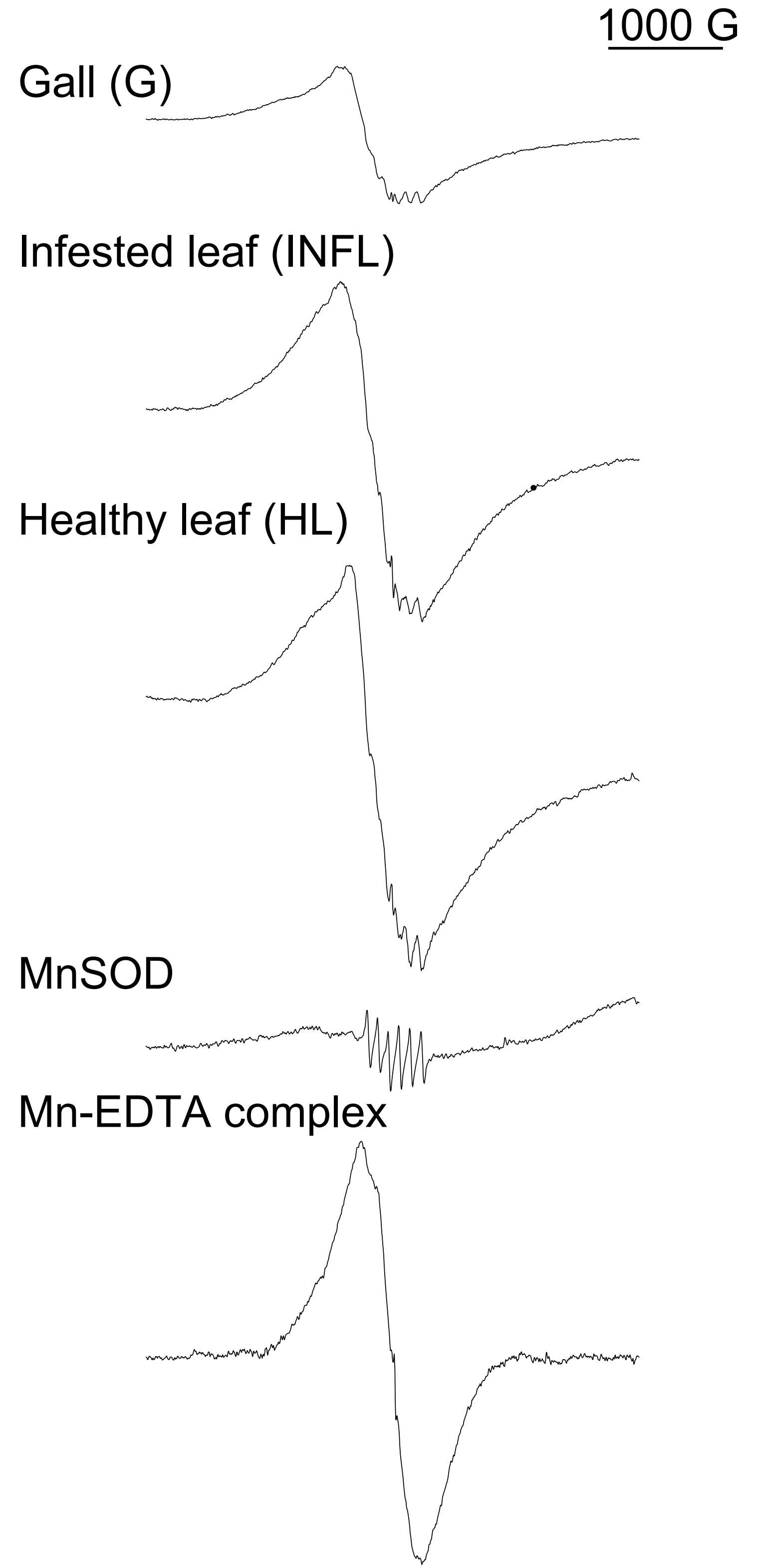
EPR application to Mn speciation in leaf galls



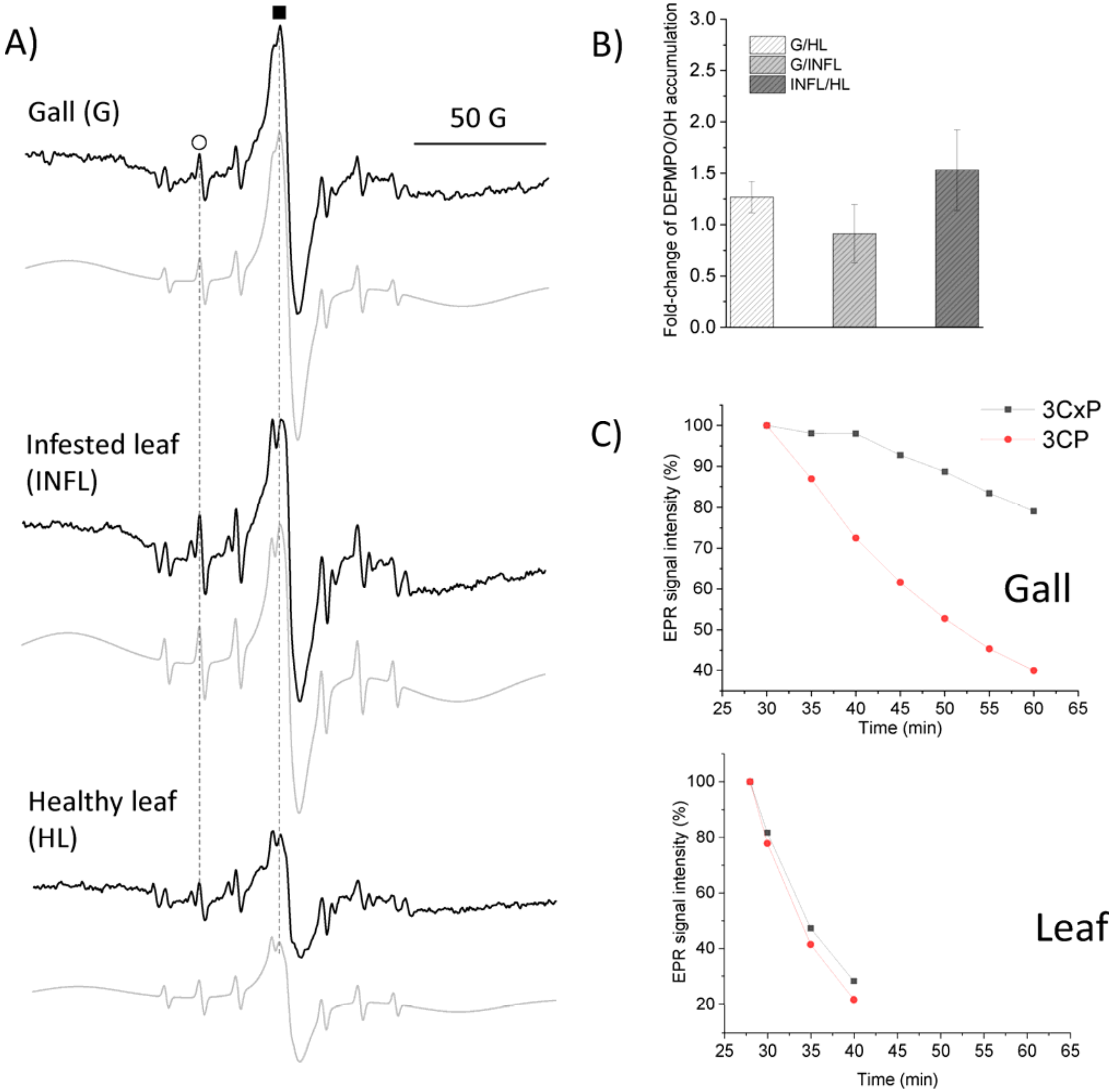
→ **EPR reveals differences in Mn binding in galls vs. leaves**

measured by room-temperature EPR
on intact leaf tissues

From: Morina F, Kuvelja A, Brückner D, Mojović M, Nakarada Đ, Bokhari SNH, Vujić B, Falkenberg G, Küpper H (2025) How eriophyid mites shape metal metabolism in leaf galls on Tilia cordata. New Phytologist 246, 2222-42
https://doi.org/10.1111/nph.70103



EPR application to ROS quenching in leaf galls



→ **EPR with spin labels reveals capacity of intact leaves and nail galls to reduce ROS**,

measured by pyrrolidine spin probes: the neutral, membrane-permeable 3CP and negatively charged membrane-impermeable 3CxP.

From: Morina F, Kuvelja A, Brückner D, Mojović M, Nakarada Đ, Bokhari SNH, Vujić B, Falkenberg G, Küpper H (2025) How eriophyid mites shape metal metabolism in leaf galls on Tilia cordata. New Phytologist 246, 2222-42 https://doi.org/10.1111/nph.70103

Introduction: NMR

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

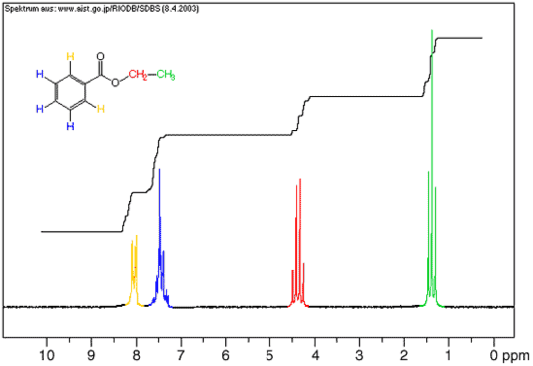
→ Substance identification

→ Purity

→ Fingerprint

→ Analysis of conformation

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



Spektrum aus: www.aist.go.jp/RIODB/SDBS (8.4.2003)

Configuration of a NMR magnet

Superconductive coil of wire in liquid helium (4 K)

Isolated by vacuum

Isolated by liquid nitrogen (77 K)

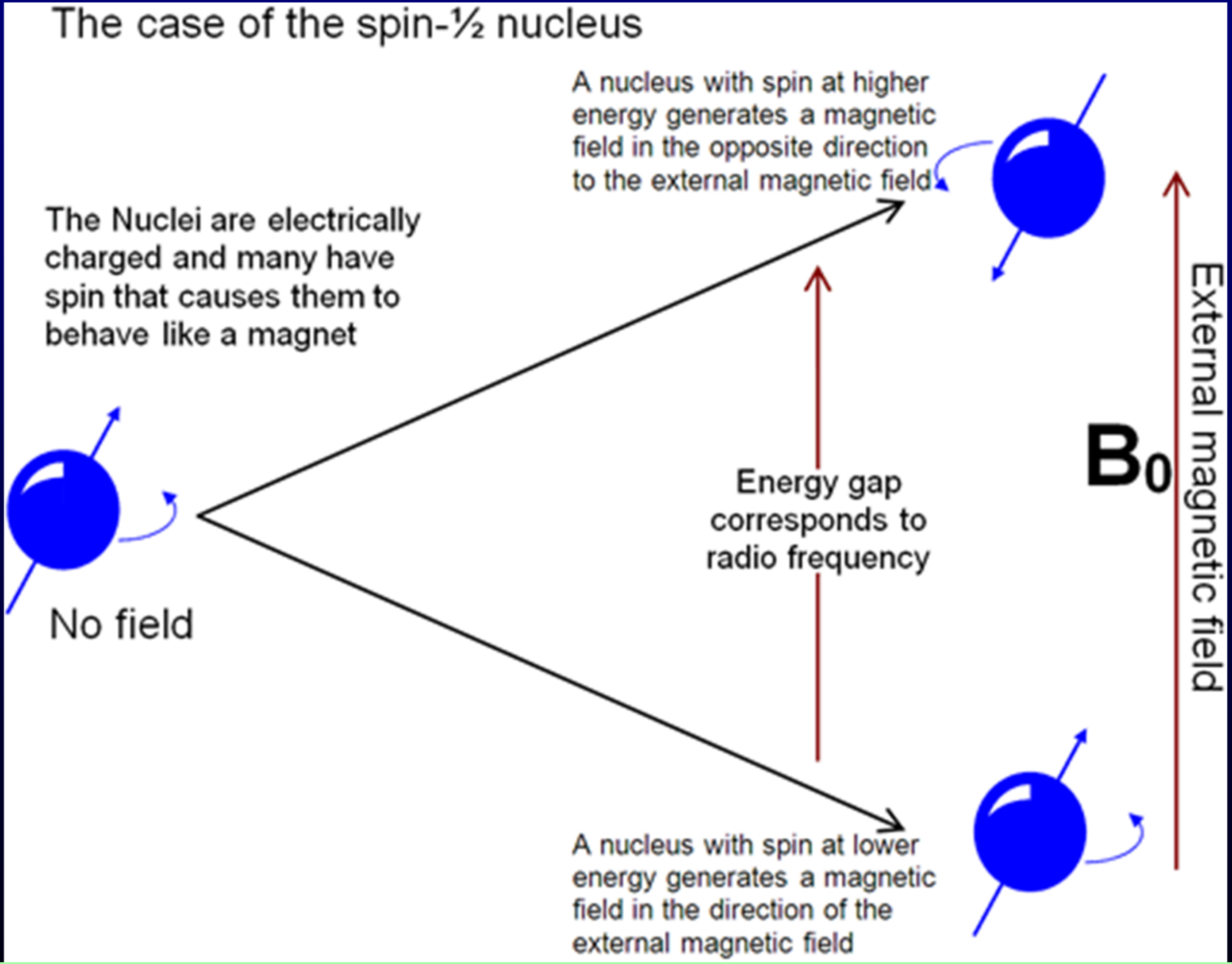
Again isolated by vacuum

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

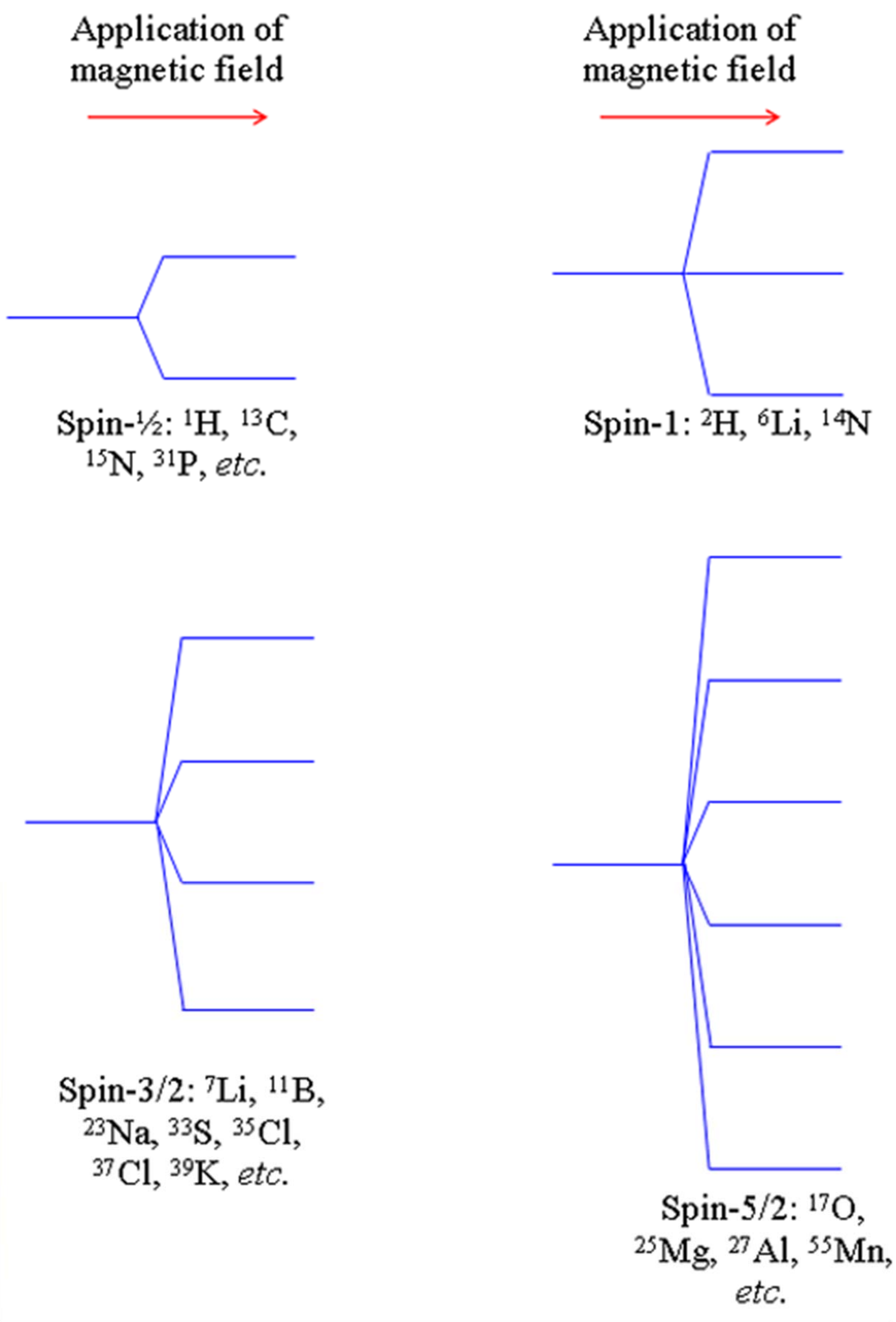


http://chem.ch.huji.ac.il/nmr/whatisnmr/whatisnmr_files/basisnmr.gif

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

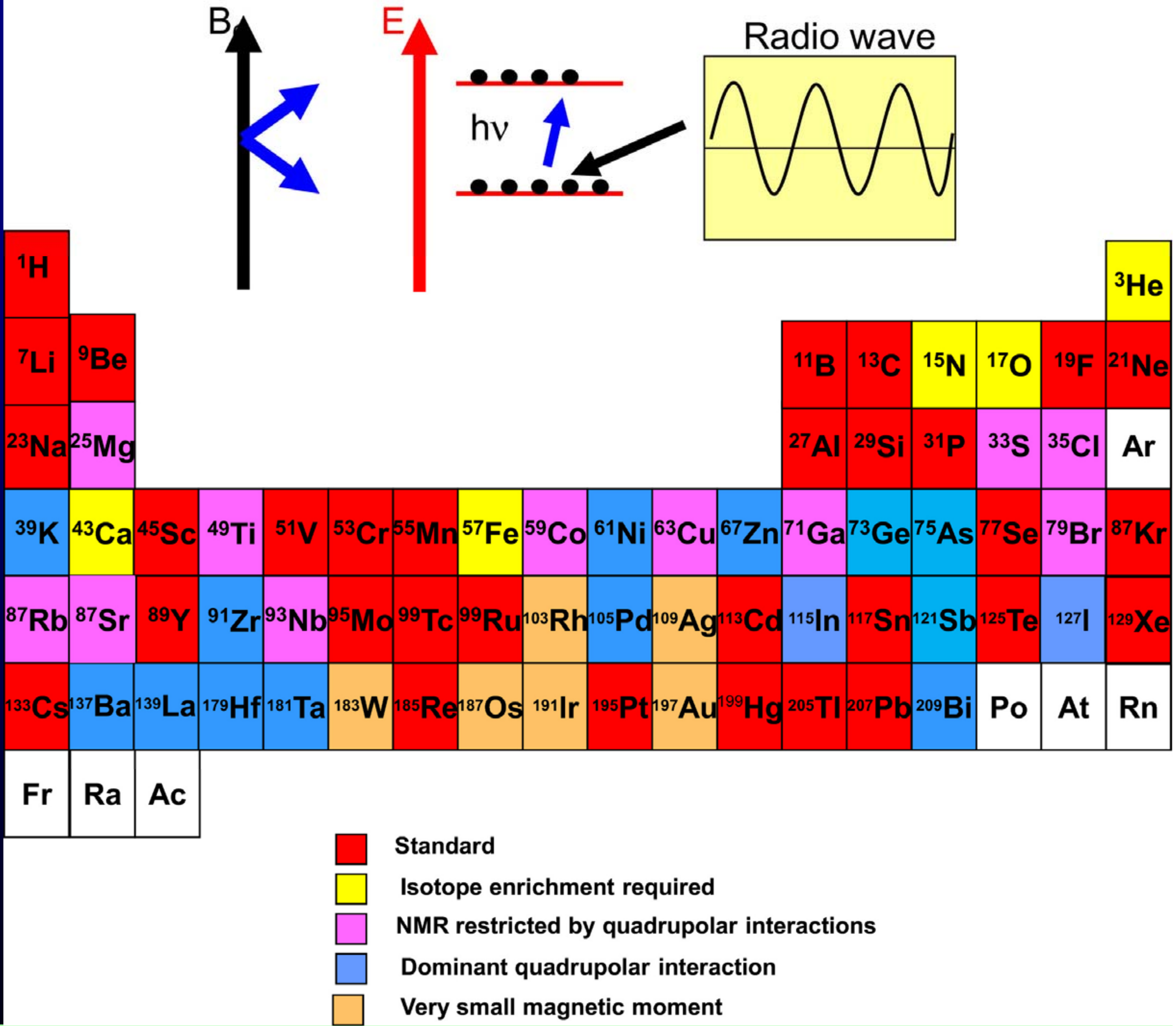


http://chem.ch.huji.ac.il/nmr/whatisnmr/quad_files/energylevels.gif

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



🟥 ^{1}H																	🟨 ^{3}He
🟥 ^{7}Li	🟥 ^{9}Be											🟥 ^{11}B	🟥 ^{13}C	🟨 ^{15}N	🟨 ^{17}O	🟥 ^{19}F	🟥 ^{21}Ne
🟥 ^{23}Na	🟪 ^{25}Mg											🟥 ^{27}Al	🟥 ^{29}Si	🟥 ^{31}P	🟪 ^{33}S	🟪 ^{35}Cl	Ar
🟦 ^{39}K	🟨 ^{43}Ca	🟥 ^{45}Sc	🟪 ^{49}Ti	🟥 ^{51}V	🟥 ^{53}Cr	🟥 ^{55}Mn	🟨 ^{57}Fe	🟪 ^{59}Co	🟦 ^{61}Ni	🟪 ^{63}Cu	🟦 ^{67}Zn	🟪 ^{71}Ga	🟦 ^{73}Ge	🟦 ^{75}As	🟥 ^{77}Se	🟪 ^{79}Br	🟥 ^{87}Kr
🟪 ^{87}Rb	🟪 ^{87}Sr	🟥 ^{89}Y	🟦 ^{91}Zr	🟪 ^{93}Nb	🟥 ^{95}Mo	🟥 ^{99}Tc	🟥 ^{99}Ru	🟧 ^{103}Rh	🟦 ^{105}Pd	🟧 ^{109}Ag	🟥 ^{113}Cd	🟦 ^{115}In	🟥 ^{117}Sn	🟦 ^{121}Sb	🟥 ^{125}Te	🟦 ^{127}I	🟥 ^{129}Xe
🟥 ^{133}Cs	🟦 ^{137}Ba	🟦 ^{139}La	🟦 ^{179}Hf	🟦 ^{181}Ta	🟧 ^{183}W	🟥 ^{185}Re	🟧 ^{187}Os	🟧 ^{191}Ir	🟥 ^{195}Pt	🟧 ^{197}Au	🟥 ^{199}Hg	🟥 ^{205}Tl	🟥 ^{207}Pb	🟦 ^{209}Bi	Po	At	Rn
Fr	Ra	Ac															

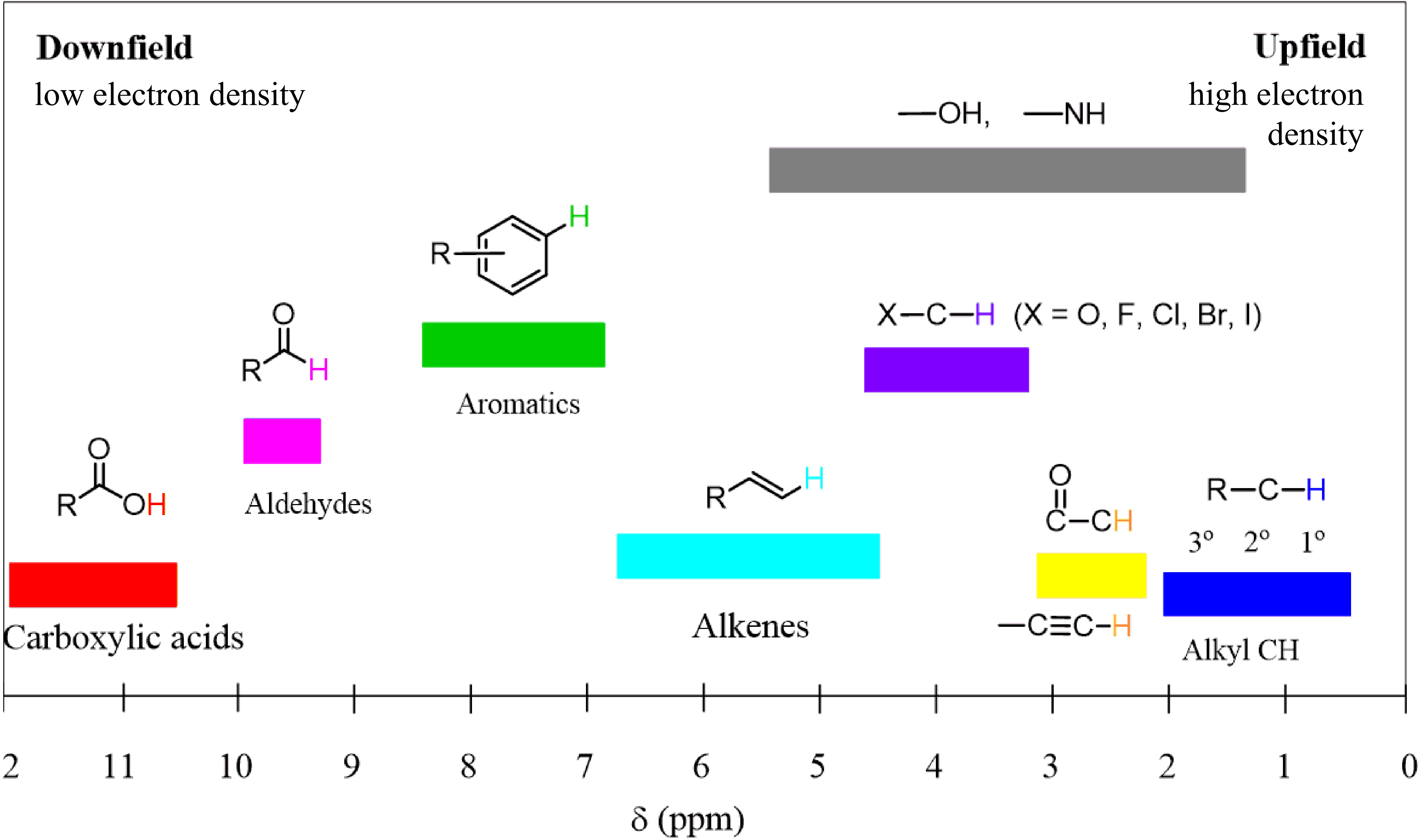
- 🟥 Standard
- 🟨 Isotope enrichment required
- 🟪 NMR restricted by quadrupolar interactions
- 🟦 Dominant quadrupolar interaction
- 🟧 Very small magnetic moment

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

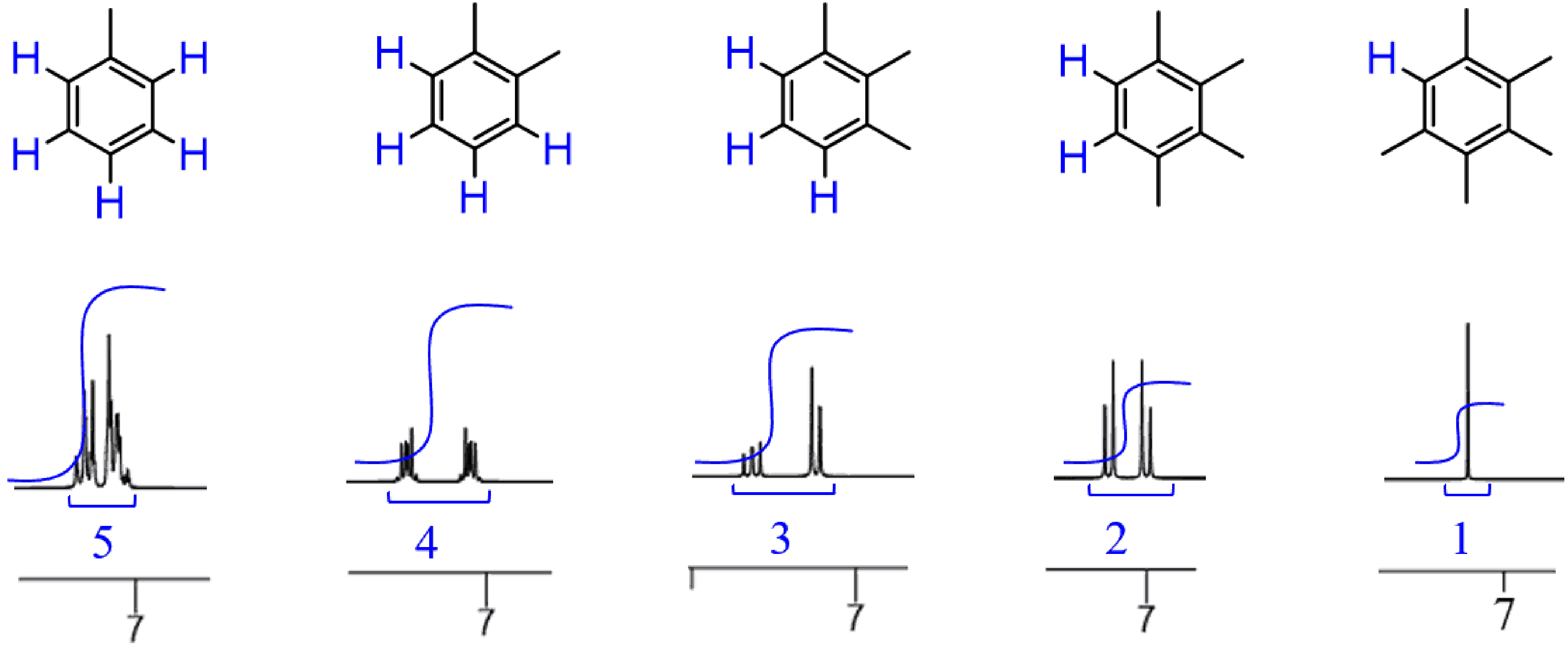
Principles

→ 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)

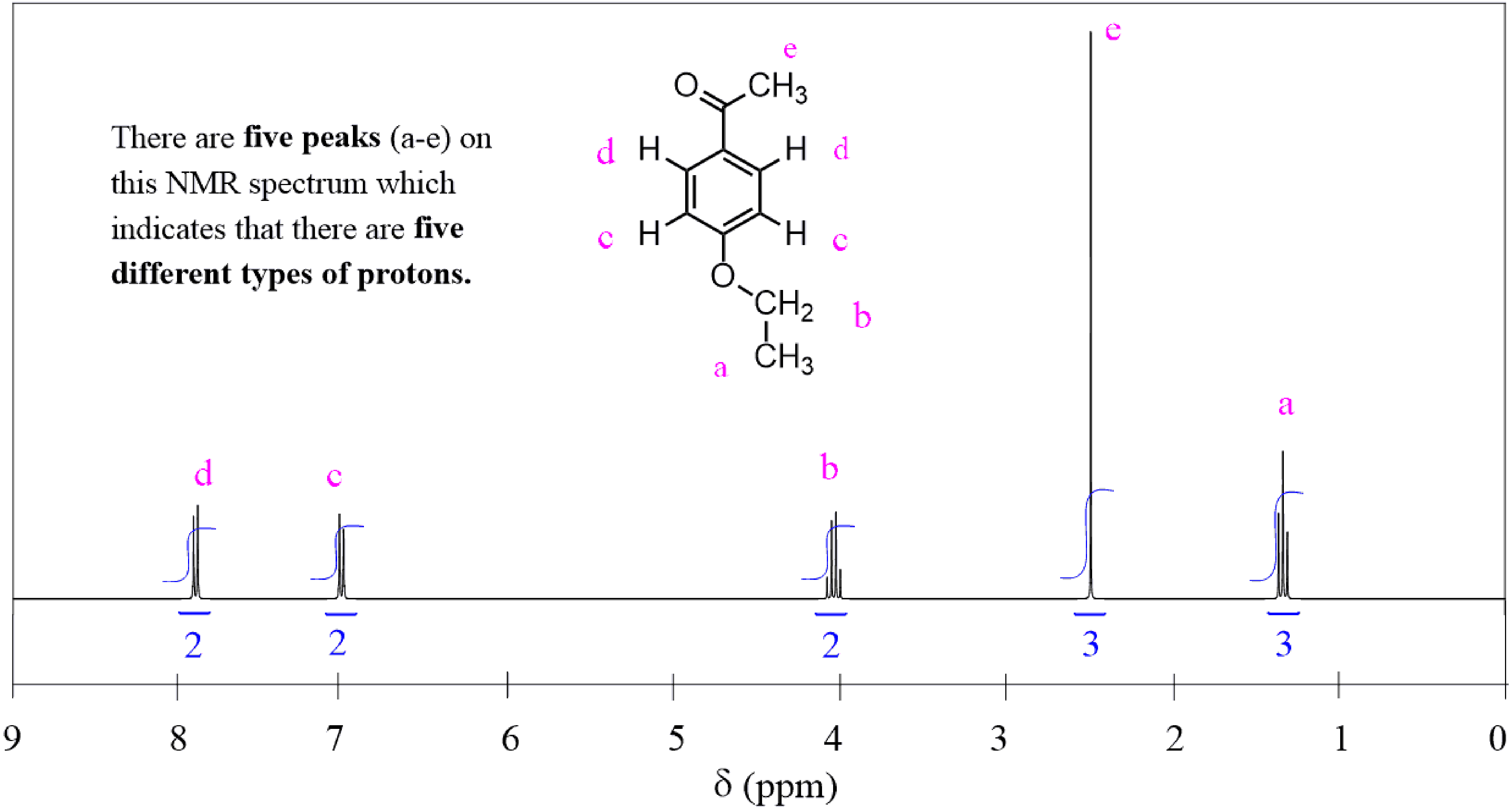


https://www.chemistrysteps.com/wp-content/uploads/2020/01/Integrals-NMR.png

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

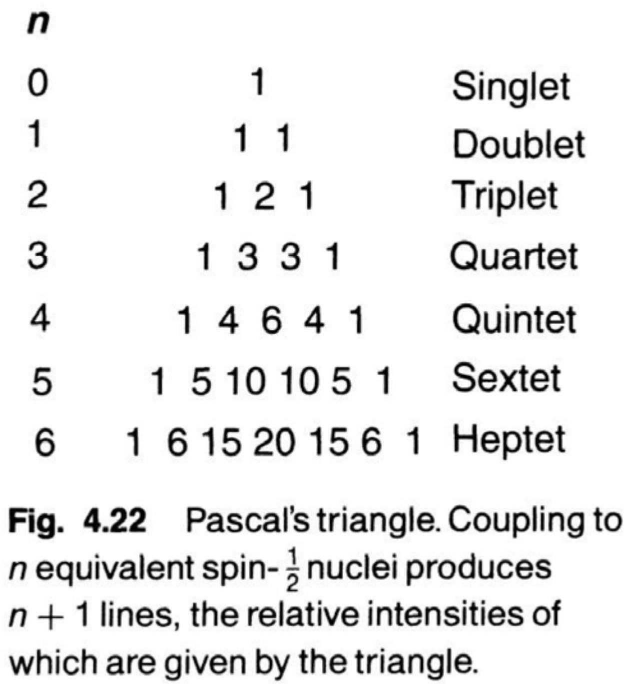
NMR: Line splitting - intensities

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.

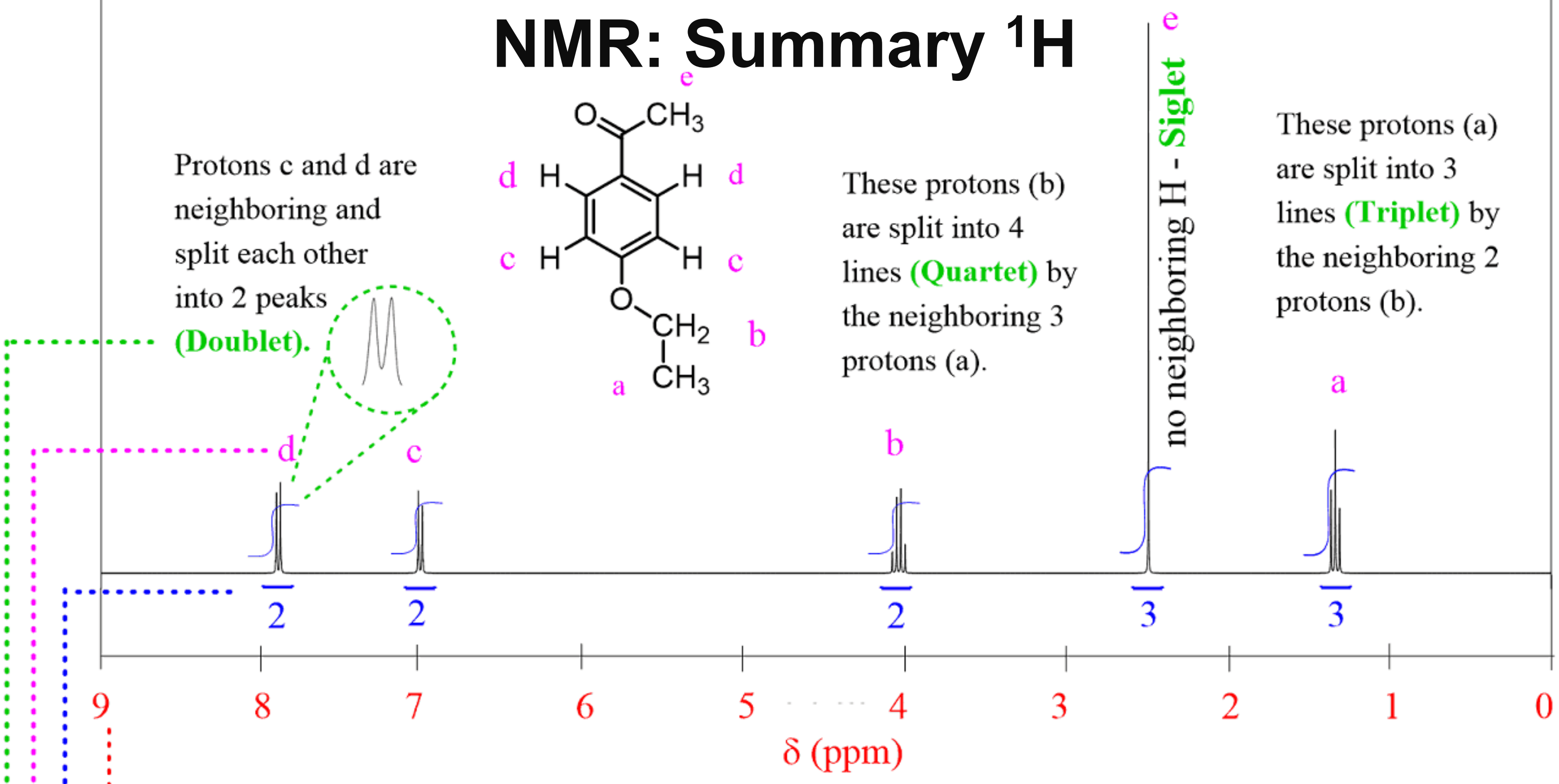
- Line intensities according to Pascal´s triangle



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 ^1H



Protons c and d are neighboring and split each other into 2 peaks **(Doublet).**

These protons (b) are split into 4 lines **(Quartet)** by the neighboring 3 protons (a).

no neighboring H - **Siglet**

These protons (a) are split into 3 lines **(Triplet)** by the neighboring 2 protons (b).

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.

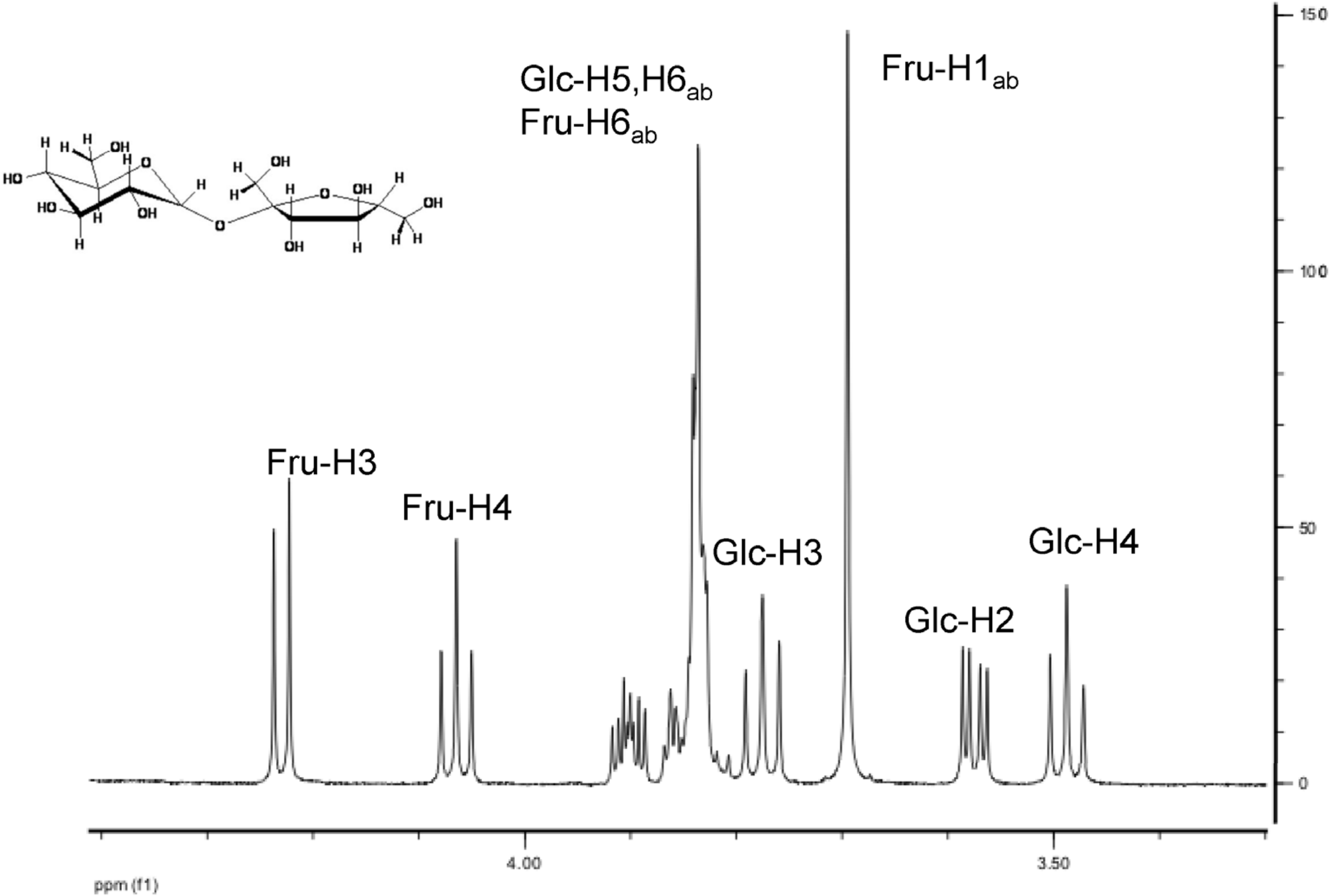
② The **number of protons** represented by each signal. This measured by the **integration** which is the surface area under each signal peak(s).

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.

https://www.chemistrysteps.com/wp-content/uploads/2020/01/Summary-of-information-we-obtain-when-looking-at-an-1H-NMR-spectrum.png

^{1}H NMR Spectrum of Glucose



Lecture slide of Peter Kroneck

^{13}C-NMR

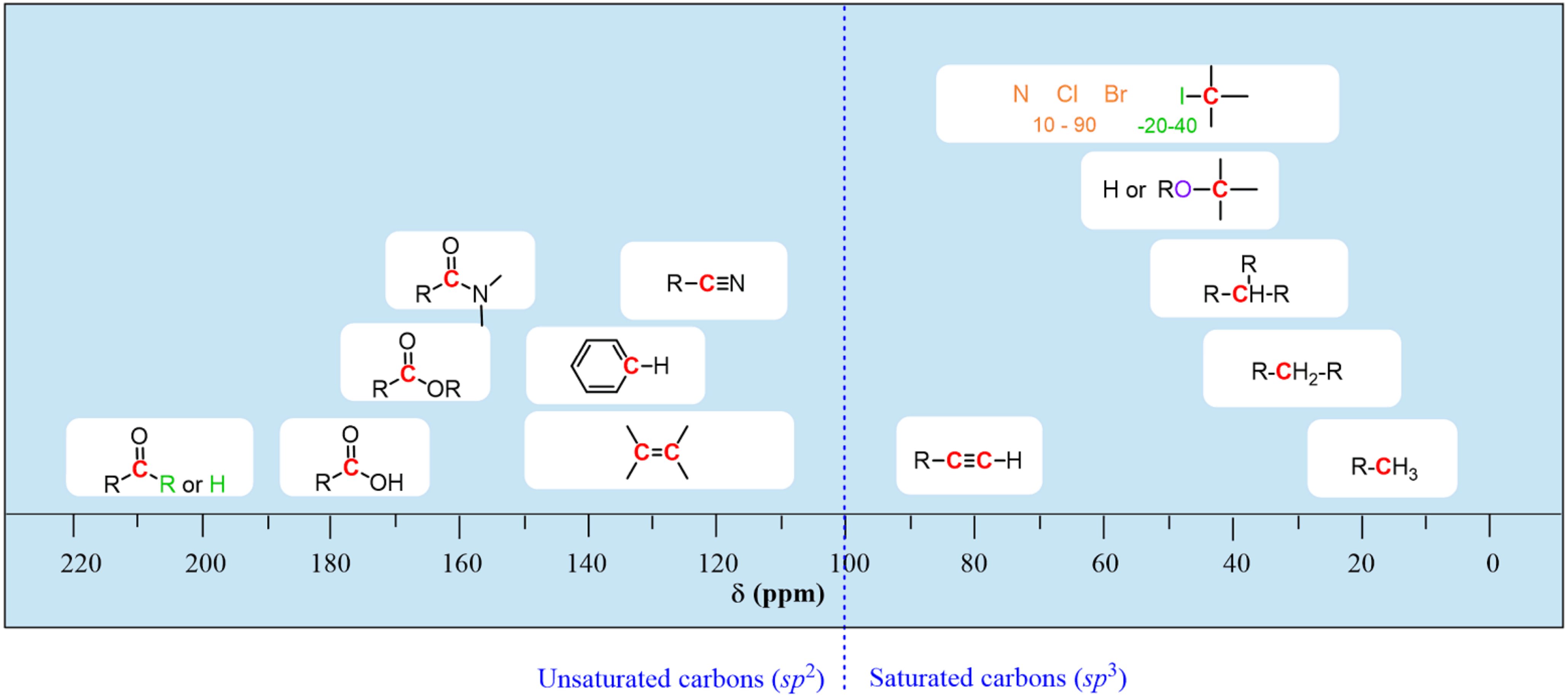
- Natural abundance of 1.1 % and low sensitivity
  → Acquisition takes more time

- 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

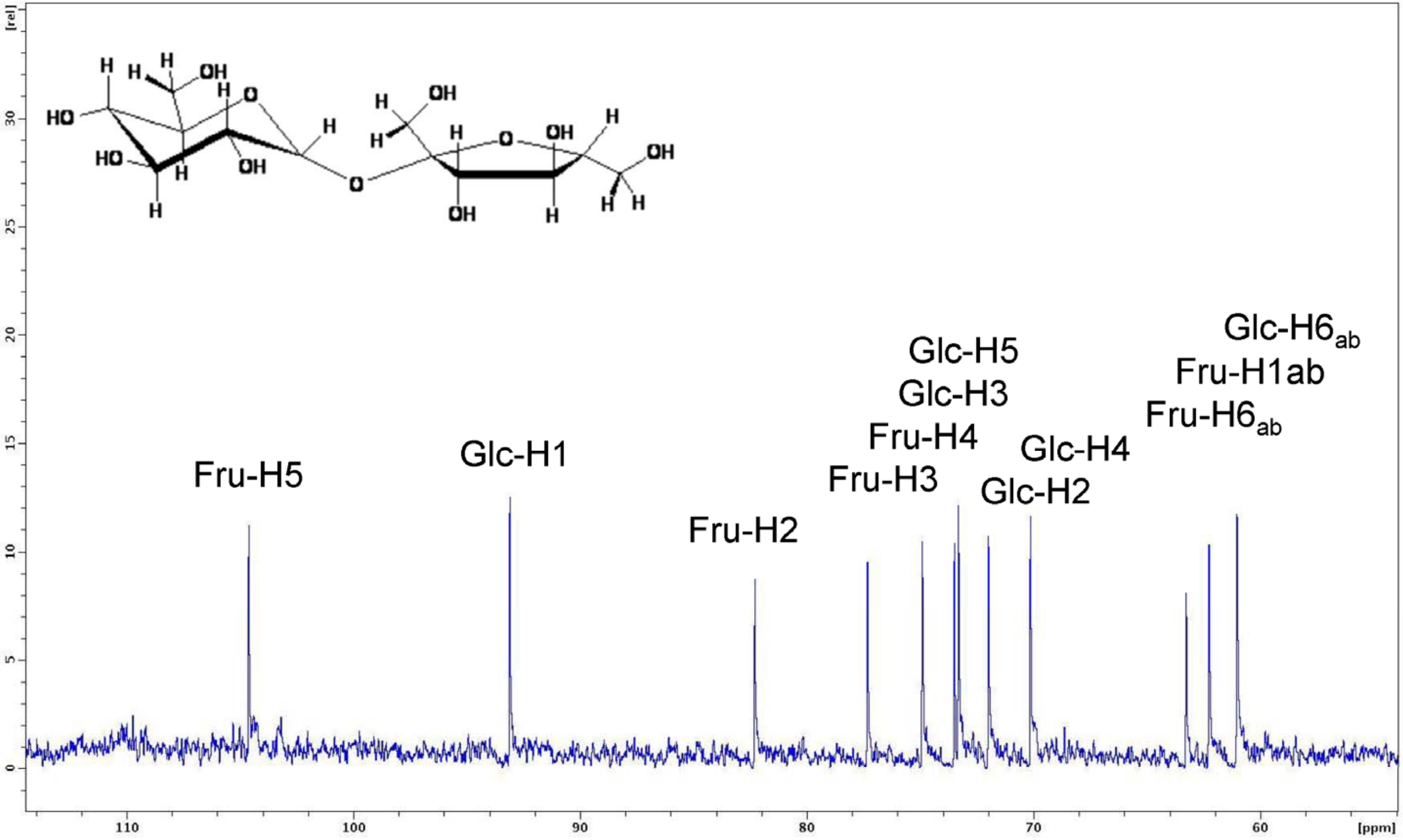
→ Determination of the number of different carbon atoms in a molecule

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



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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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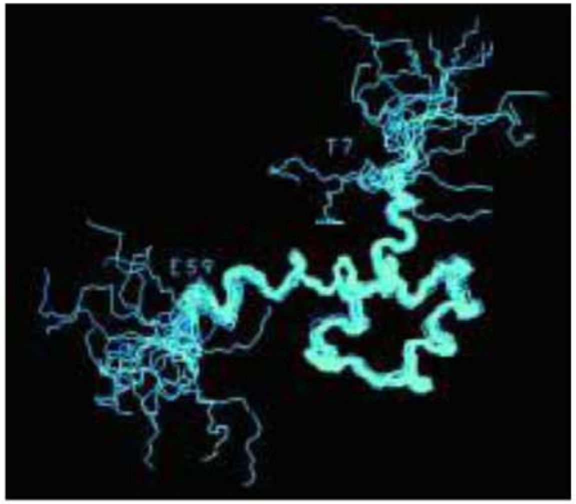
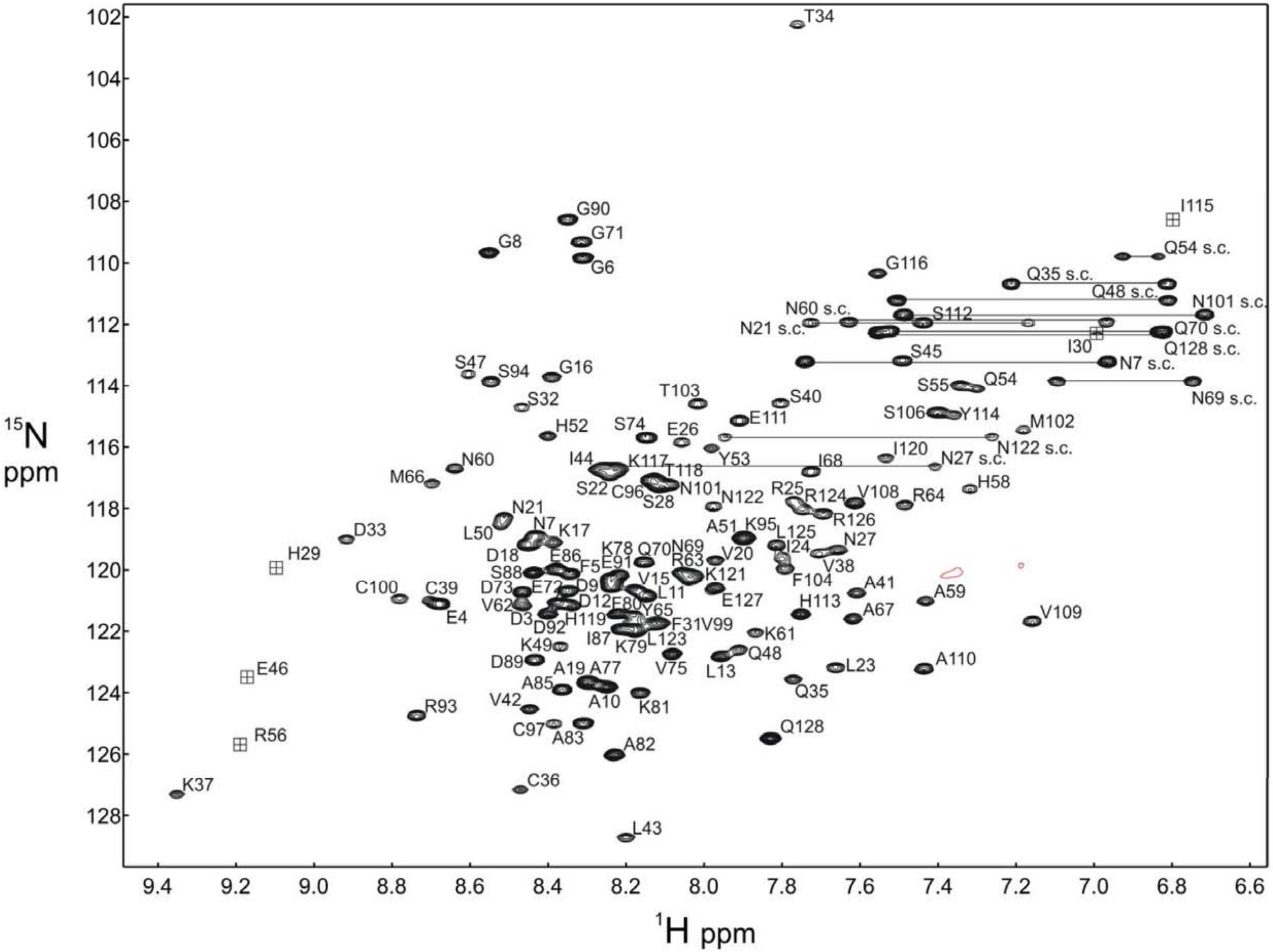


Figure 1. NMR structure of the *Antennapedia* homeodomain. A bundle of 20 superimposed conformers represents the polypeptide backbone. For the polypeptide segment 7–59 the tight fit of the bundle indicates that the structure is defined with high precision, whereas the two chain ends are disordered.

- 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 ^{1}H with ^{13}C or ^{15}N NMR ("2-dimensional NMR")



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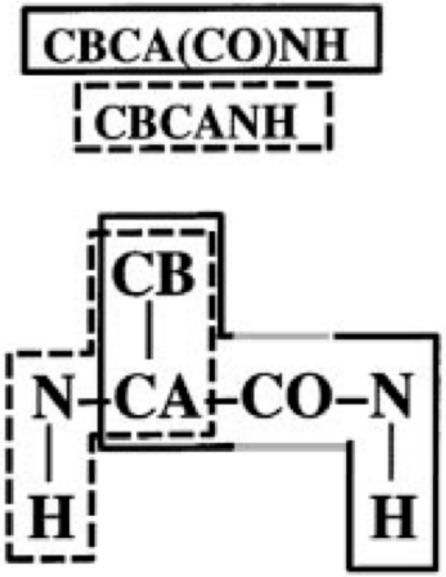
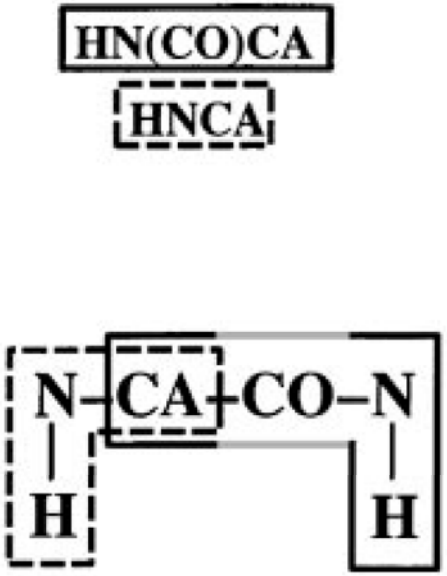
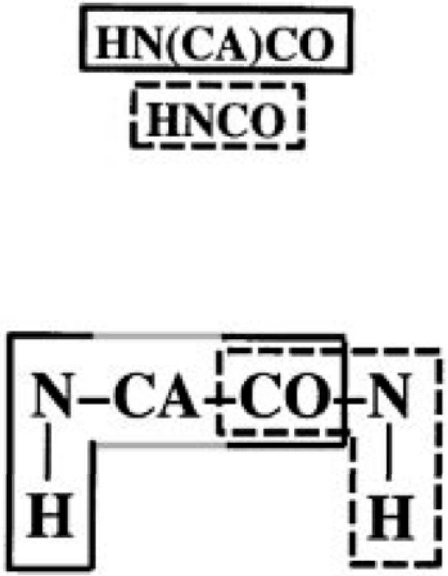
NMR Application for resolving protein structures (III) → Structure assignment (1)

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







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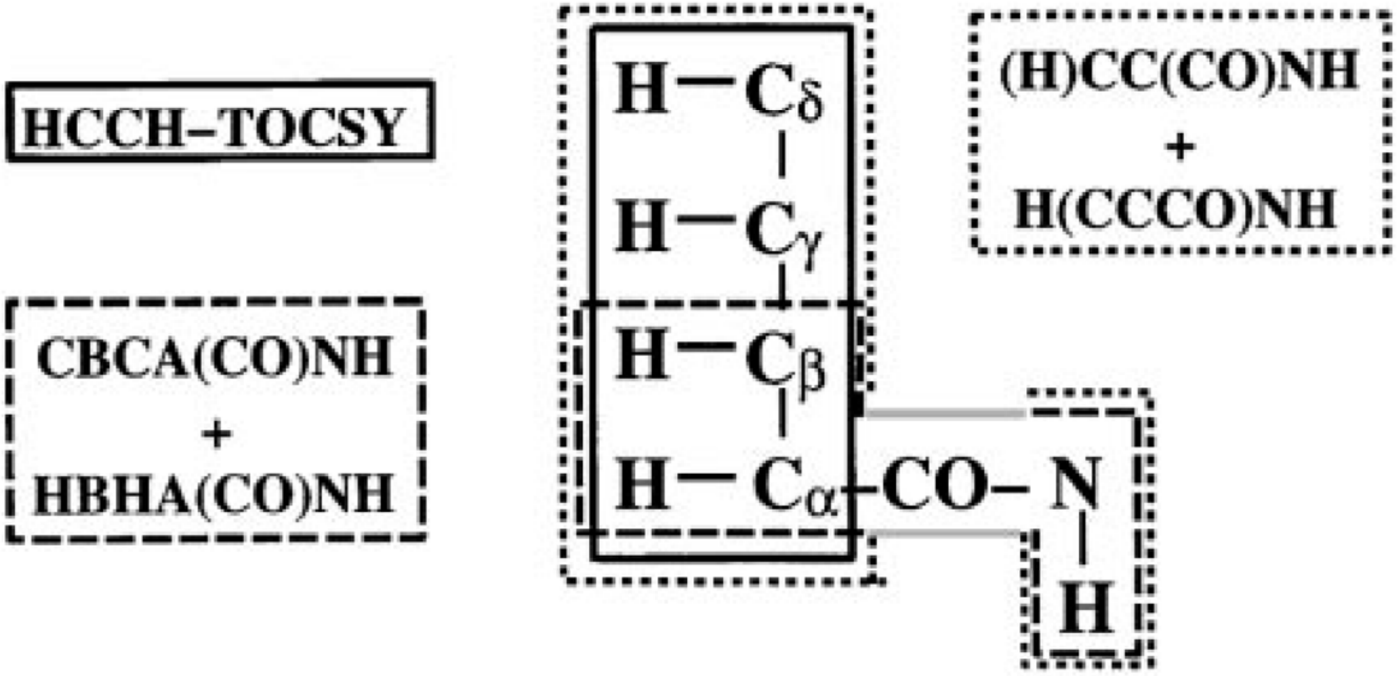
NMR Application for resolving protein structures (IV) → Structure assignment (2)

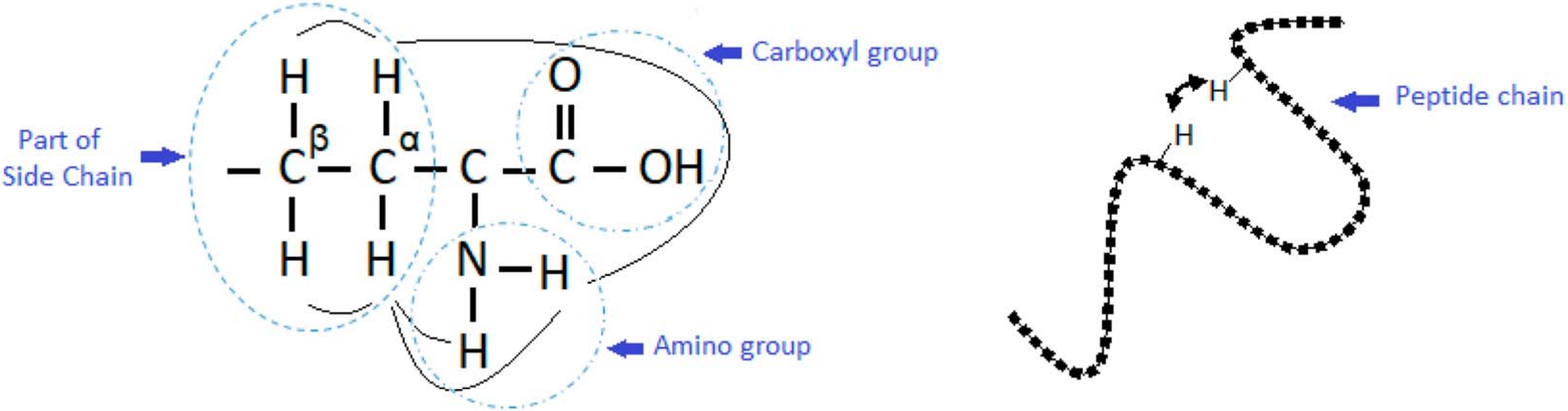
- Side chain assignment: determine all relevant chemical shifts of ^{1}H, ^{13}C and ^{15}N



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.



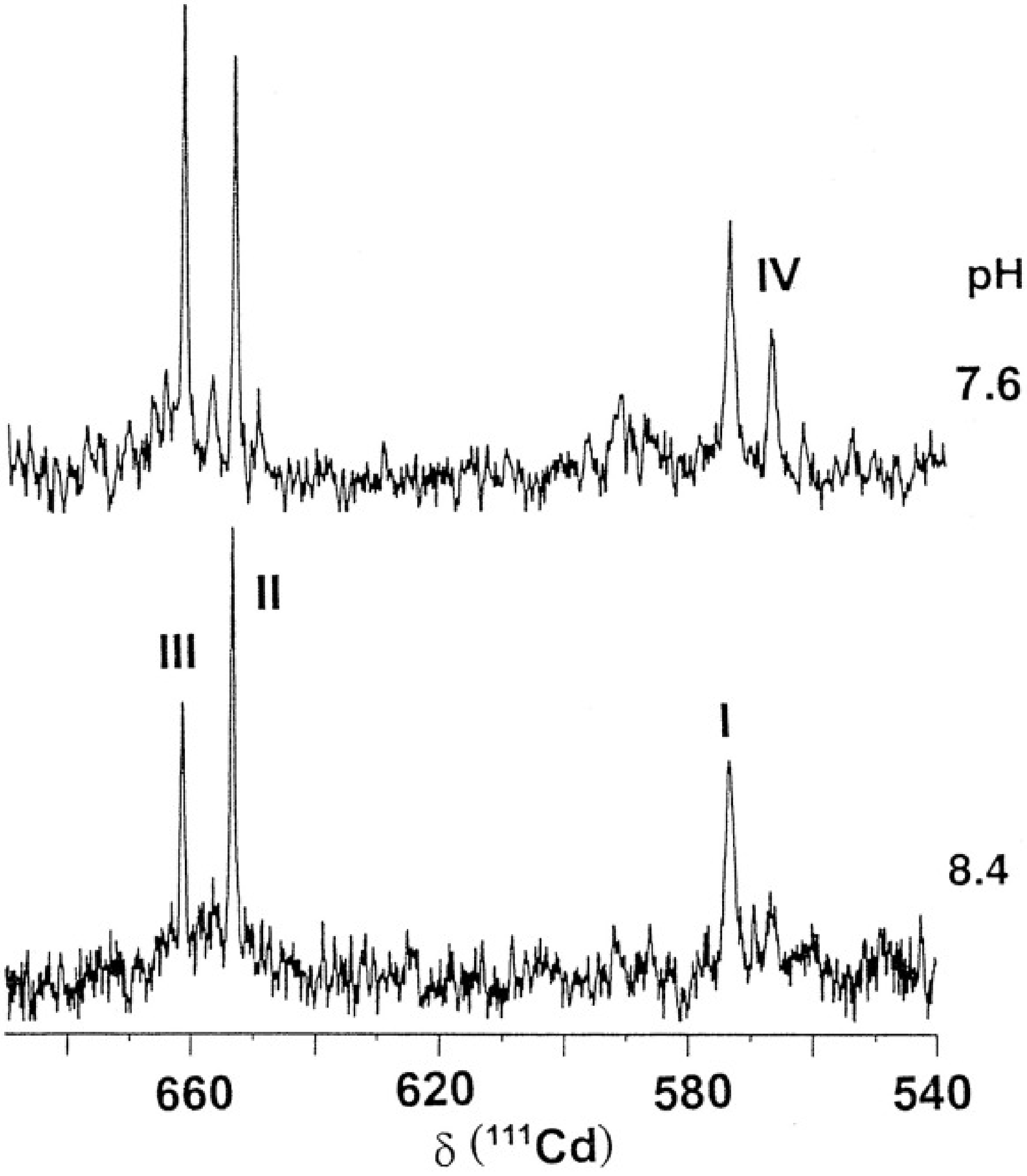
Through bound (scalar) coupling (a); through space coupling—nuclear Overhauser effect (NOE) (b).

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 CdS_4 coordination, and peak I (572 ppm) is in the range for $CdNS_3$ or CdN_2S_2 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