

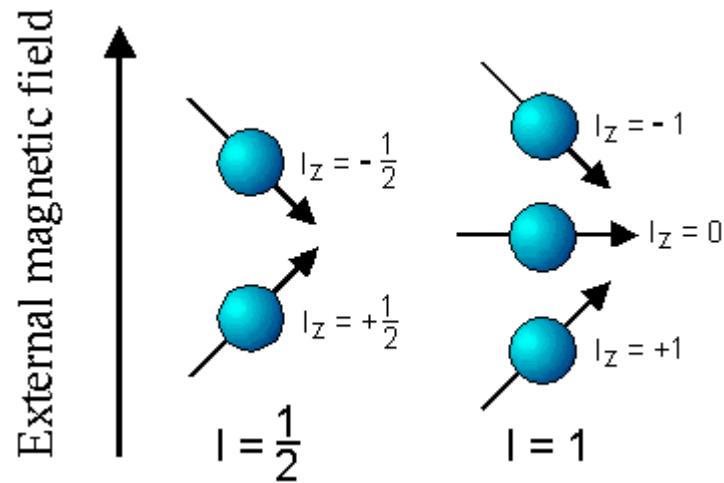
C6200–Biochemické metody

11A\_NMR A EPR

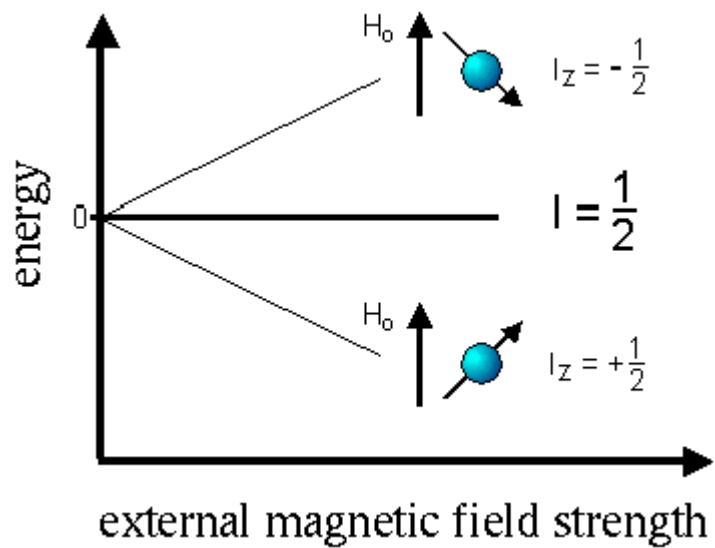
Petr Zbořil

# Nukleární magnetická rezonance

- Jádra vykazující nenulový jaderný spin (závisí na počtu protonů a neutronů) se orientují v externím magnetickém poli.
- Spin může nabývat několika orientací v závislosti na své hodnotě:
- Počet =  $2I + 1$ , kde  $I$  je spinové kvantové číslo o hodnotách  $0, \frac{1}{2}, 1, \dots$
- Hodnoty  $\in I - 1 \in -3/2, -1, -1/2, 0, \frac{1}{2}, 1, 3/2 \dots$



# Nukleární magnetická rezonance



# Nukleární magnetická rezonance

Velikost magnetického momentu  $M$ , gyromagnetický poměr  $\gamma$ , jaderný magneton  $\mu$ , Planckova konstanta ( $\hbar = h/2\pi$ ) a rozlišovací faktor  $g_I$  a hmotnost jádra  $m$  a jeho náboj  $e$  jsou ve vztahu

$$M = \gamma \cdot \hbar \cdot \sqrt{I(I+1)}$$

$$\gamma = \mu \cdot g_I / \hbar$$

$$\mu = e \cdot \hbar / 2m$$

A ten určuje energii jednotlivých orientací v magnetickém poli intenzity  $B_0$

$$E_I = - M_z \cdot B_0 + E_0$$

$$M_z = \gamma \cdot \hbar \cdot m_I$$

Kde magnetické kvantové číslo  $m_I \equiv I, (I-1) \dots -(I-1), -I$ , počet  $2I+1$

$$\text{Pro } I = \frac{1}{2}, \quad m_I = \pm \frac{1}{2}$$

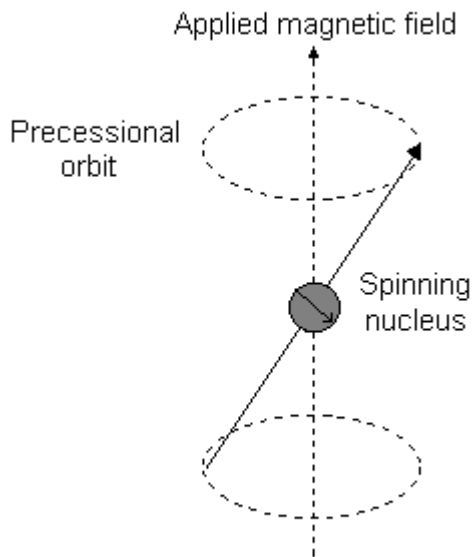
a energetický rozdíl mezi jednotlivými stavy (orientacemi vektoru spinu) je

$$\Delta E = \mu \cdot g_I \cdot B_0$$

# Nukleární magnetická rezonance

Magnetický moment rotujícího jádra  $\mu$

$$\mu = \gamma \hbar / 2\pi \quad \gamma \text{ gyromagnetický poměr}$$



Umístění rotujícího magnetu v elektrickém poli

$$\text{Frekvence rotace: } \nu_0 = \gamma B_0$$

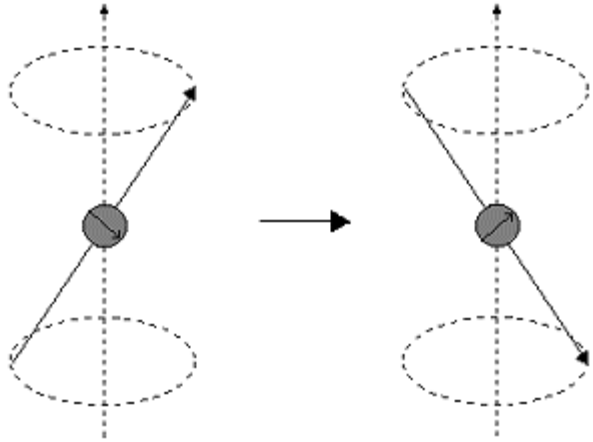
$B_0$  – indukce magnetického pole

Larmorova frekvence

H – 200 MHz pro 4,7 T atd.

Označení přístrojů – 200, 300, atd.

# Nukleární magnetická rezonance



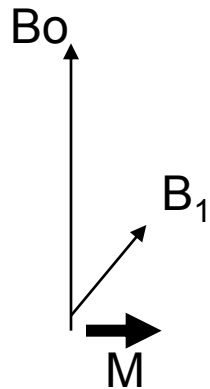
Rozdíl mezi dvěma energetickými hladinami

$$\Delta E = \gamma h B_0 = h \nu_0$$

pokud působíme elm. polem o frekvenci  $\nu_1 = \nu_0$

Dojde k rezonanci a přechodu na vyšší Hladinu

$$\nu_0 = \gamma B_0$$



# Nukleární magnetická rezonance (B = 2,3 T)

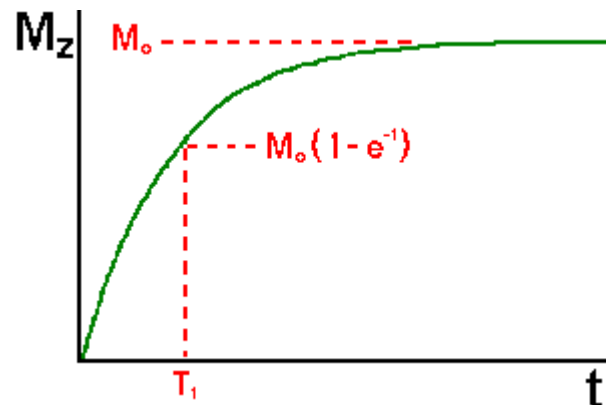
Jádro	I	$\nu_0$ (MHz)	citlivost	% zastoupení
1H	$\frac{1}{2}$	100	1	99,9
13C	$\frac{1}{2}$	25	0,016	1
15N	1	10	0,001	0,36
19F	$\frac{1}{2}$	94	0,8	100
31P	$\frac{1}{2}$	40	0,067	100
17O	$\frac{5}{2}$	13	0,003	0,04

# Nukleární magnetická rezonance

## Spin-mřížková relaxace

Jádro v excitovaném stavu se zbavuje energie relaxací

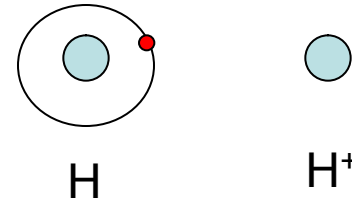
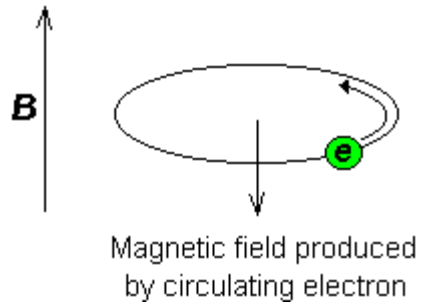
Relaxační čas (střední doba života jader v excitovaném stavu) –  $T = 10^{-1} - 10^2 \text{s}$





# Nukleární magnetická rezonance

## Stínění jádra – chemické posuny



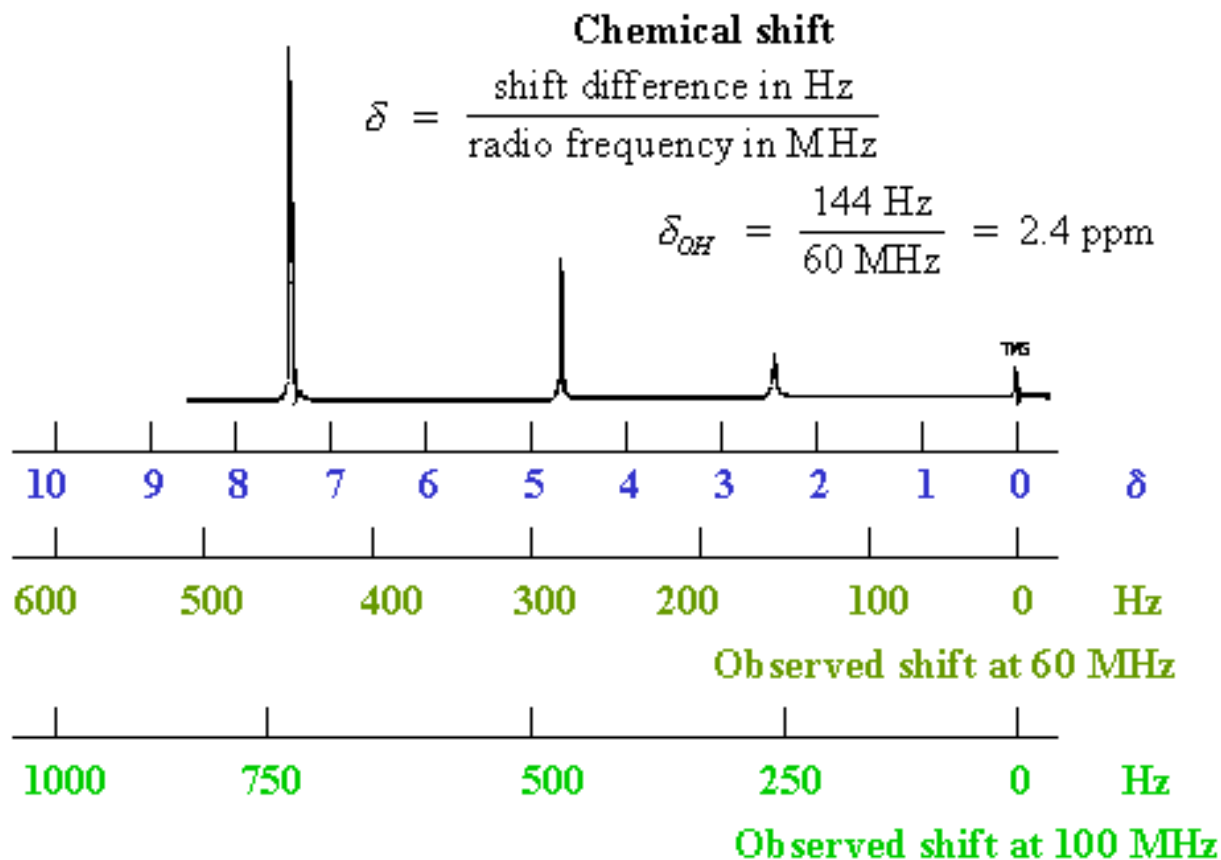
$$B = B_0 - \sigma B_0$$

$$\sigma = (B - B_0) / B_0 \text{ (x } 10^6 \text{)}$$

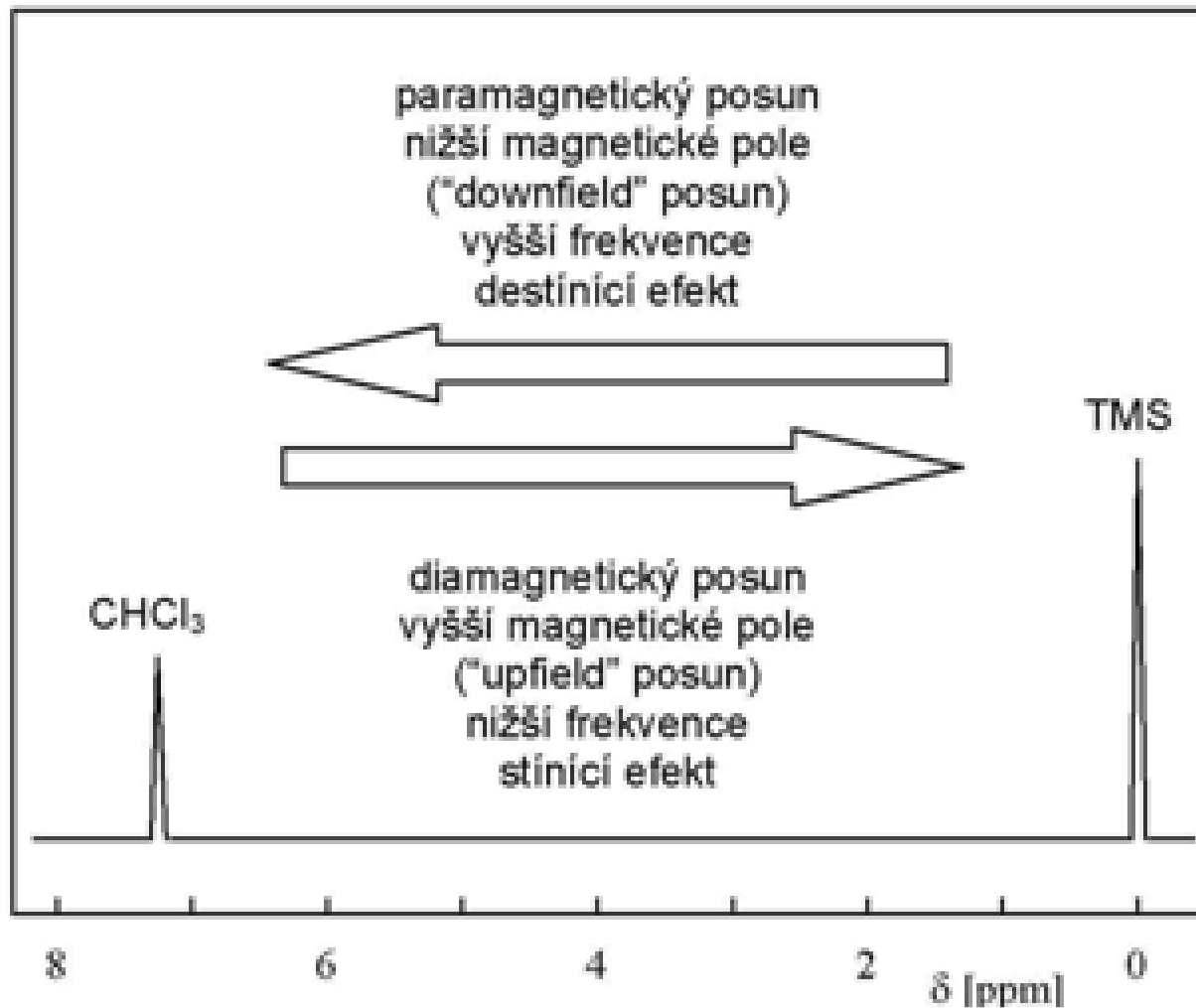
$$\delta = 10 - \sigma$$

$\delta$  chemický posun (ppm)

# Chemický posun



# Stínění a chemický posun



# Standart pro $^1\text{H}$ -NMR

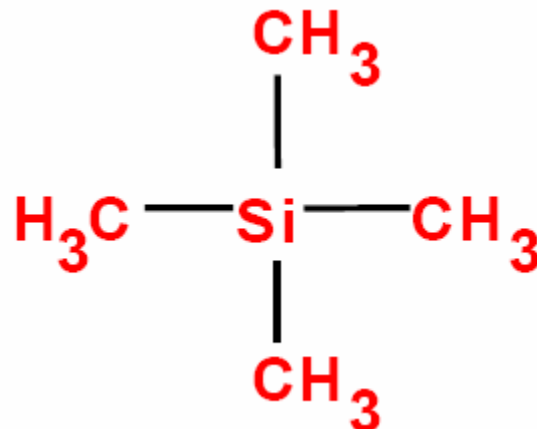
Tetrametylsilan

- jediná  $^1\text{H}$  linie snadno  
rozpoznatelná

- singletový signál nejvíce vpravo

- přidává se přímo k vzorku

- po skončení měření ho lze mírným  
zahřátím ze vzorku odstranit



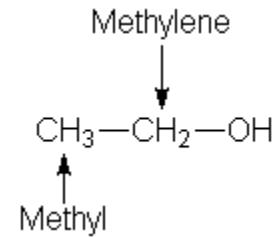
# Nukleární magnetická rezonance

## Chemické posuny

CH <sub>3</sub>	1
CH <sub>2</sub>	1,5
R <sub>2</sub> NH	0,5-2
RNH <sub>2</sub>	1,5
ArCH <sub>3</sub>	2,5
-C≡CH	3
Ar-NH <sub>2</sub>	3,5 – 4,5
X-CH <sub>3</sub>	2,5 – 3,5
R-O-CH <sub>3</sub>	4
=C=CH <sub>2</sub>	5
ArH	7-8
RCOH	10
RCOOH	10-12

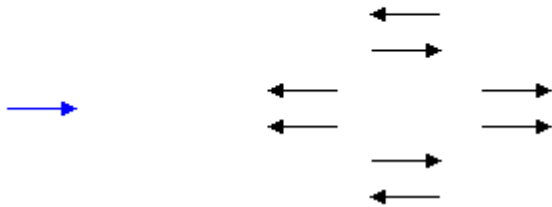
# Nukleární magnetická rezonance

## Multiplicita signálu – spin-spinová interakce



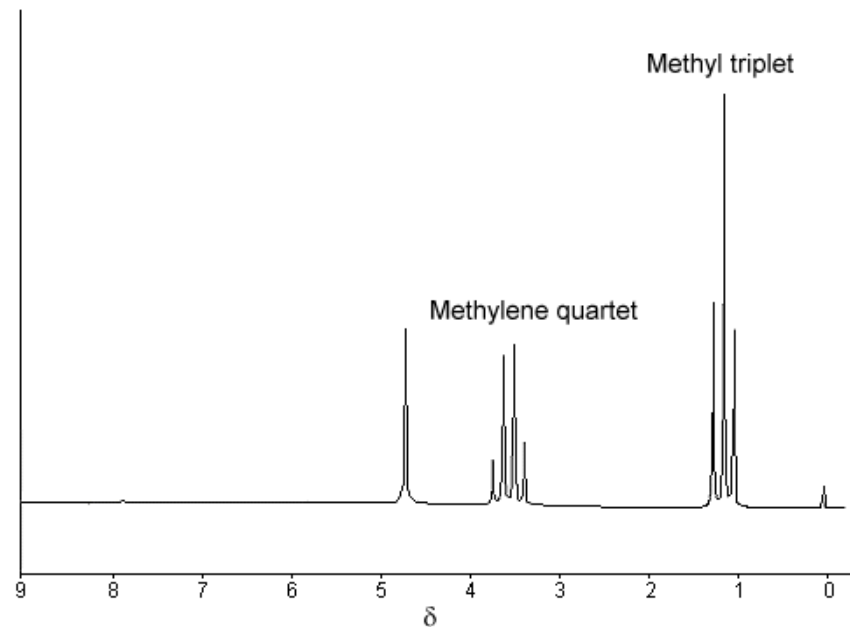
Applied field

Spin orientations of methylene protons



Multiplicita =  $n+1$

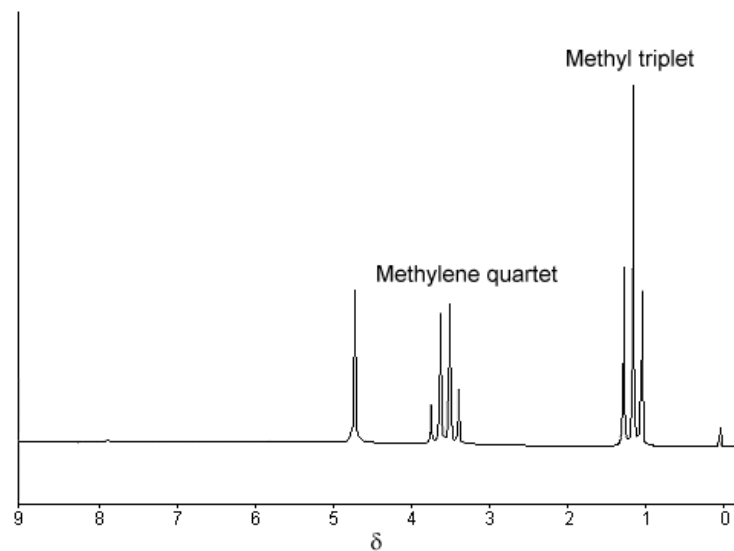
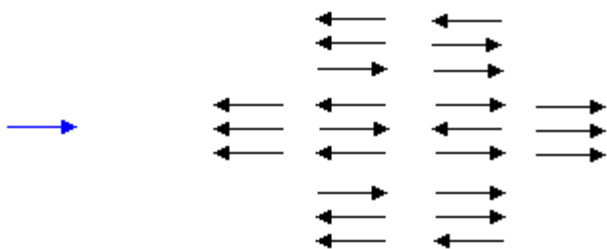
$(x + 1)^n$



# Nukleární magnetická rezonance

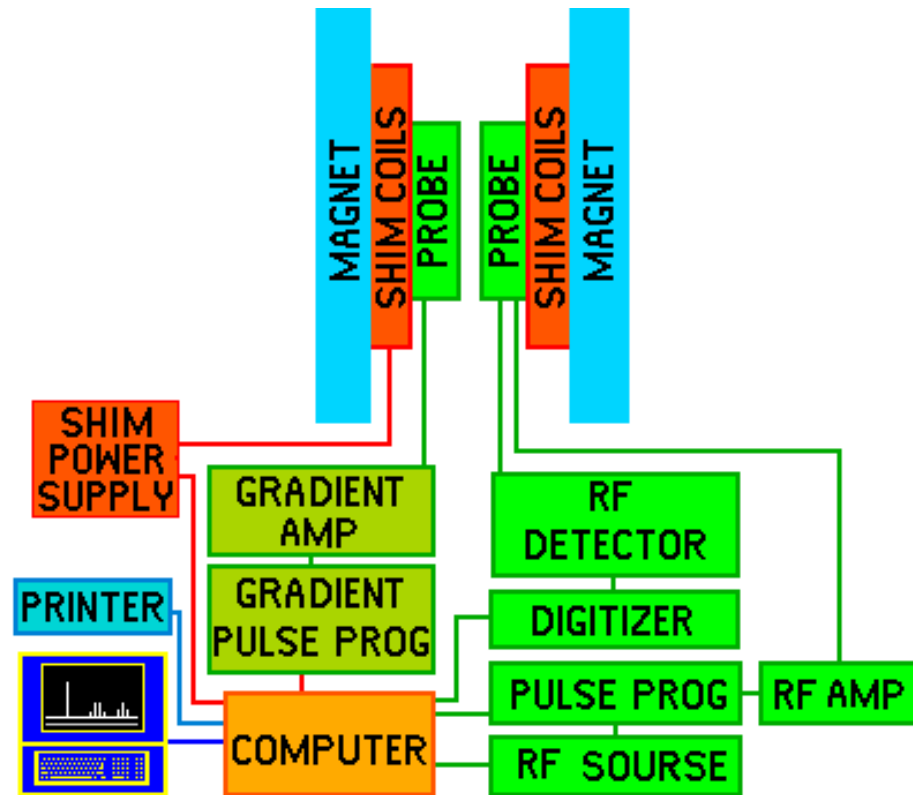
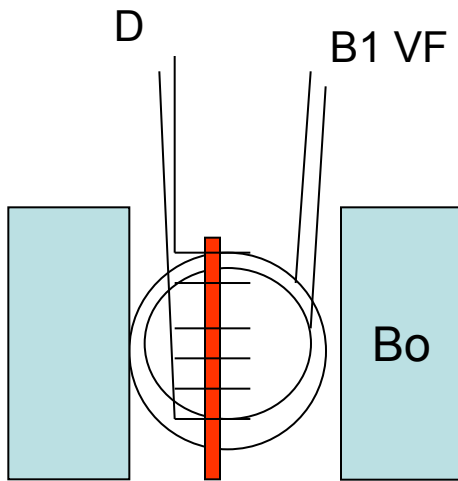
Applied field

Spin orientations of methyl protons



# Nukleární magnetická rezonance

## Přístrojové vybavení





# Využití NMR

Struktura a její změny

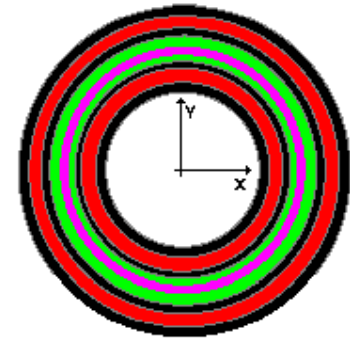
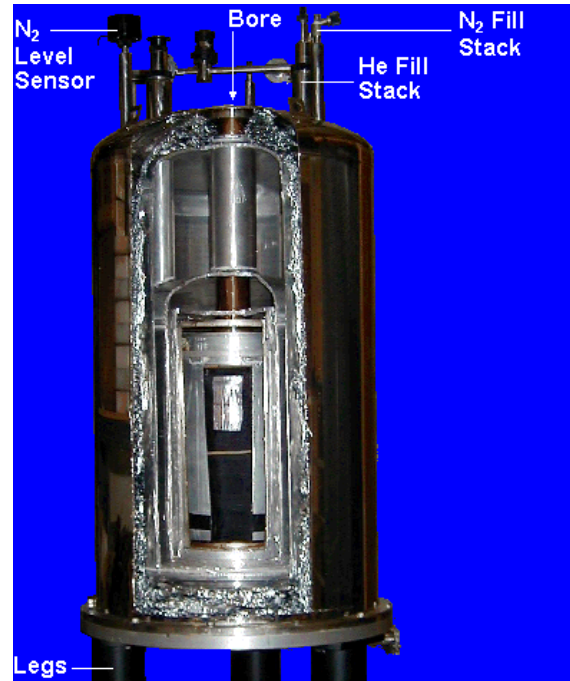
**chemický posun  $\delta$**  – chemické okolí jádra, typ skupiny

**Multiplicity a interakční konstanty J** - počet sousedních mag. aktivních jader a geom. uspořádání

**Integrální intenzity signálů** - počet ekvivalentních atomů v molekule

Korelační signály atp.

# Nukleární magnetická rezonance

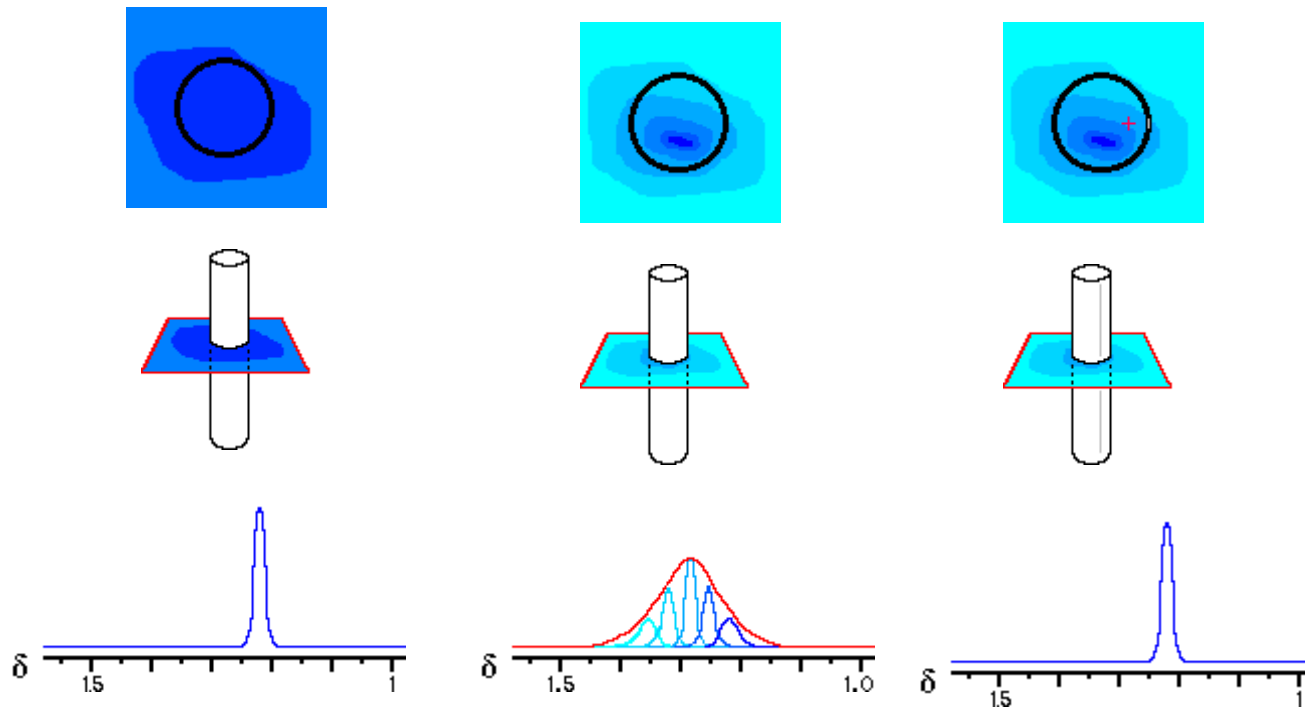


- Vacuum
- Liquid Helium
- Liquid Nitrogen
- Container & Support
- Superconducting Coil

Supravodivý magnet 7 T

# Nukleární magnetická rezonance

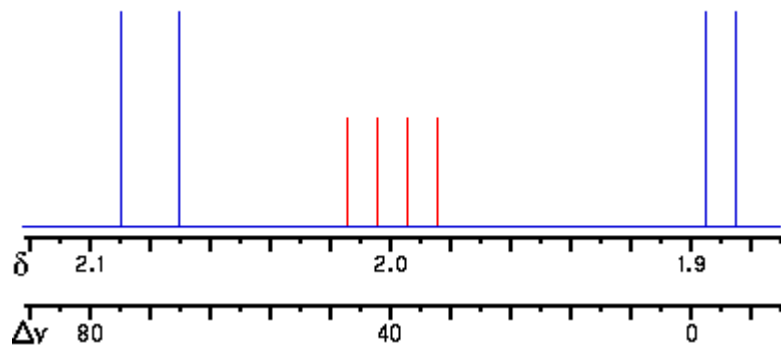
Vzorek – homogenizace pole



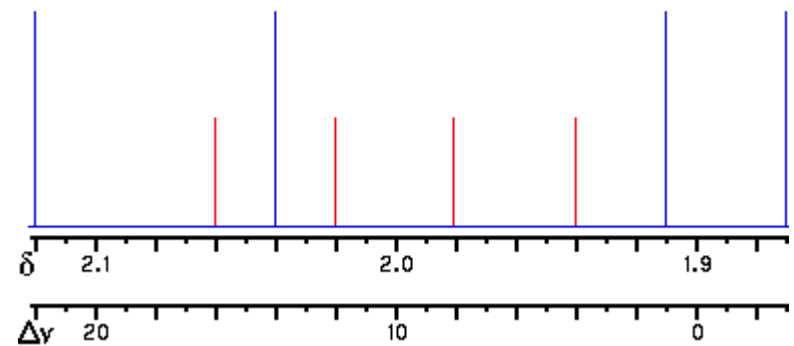
# Nukleární magnetická rezonance

Volba přístroje

400 MHz



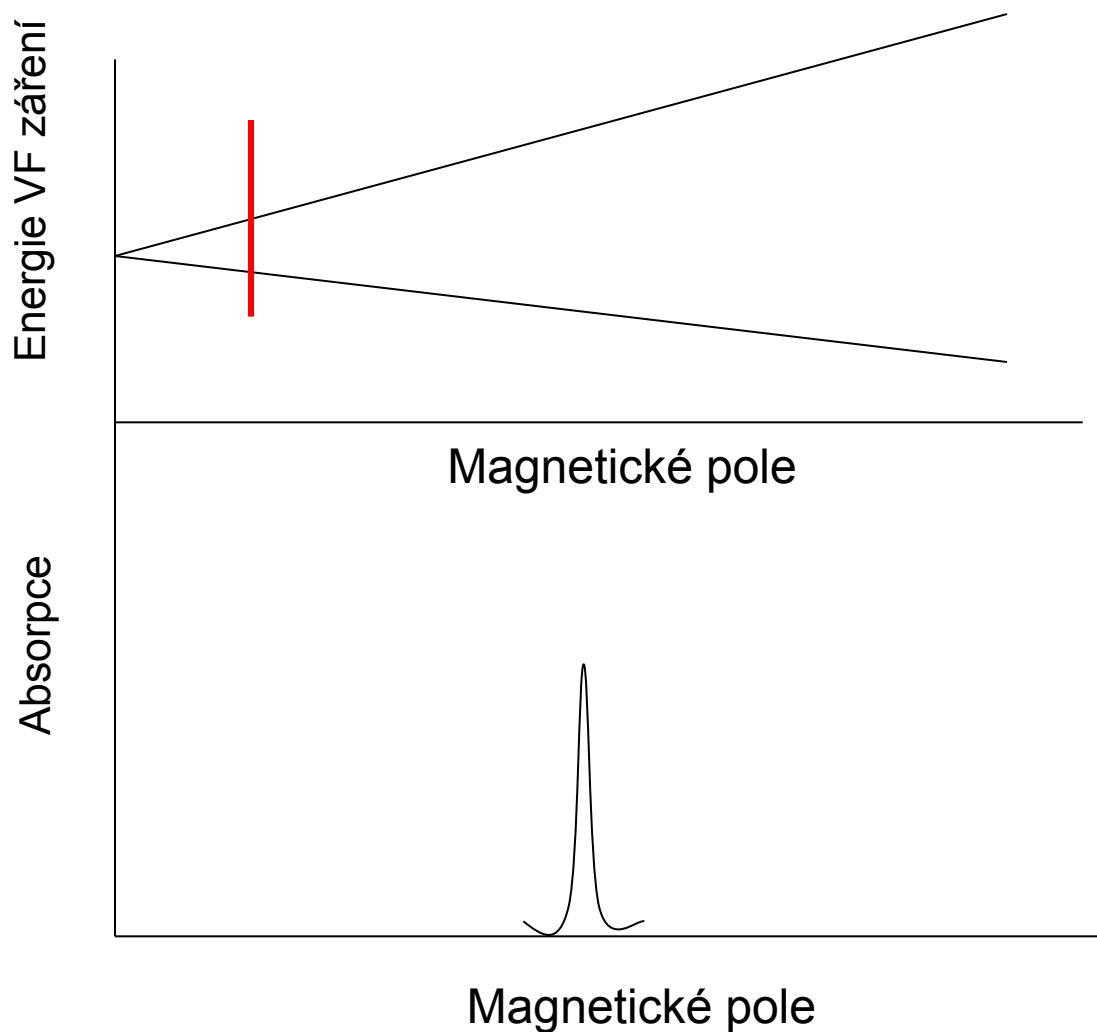
100 MHz



Atomy: 1,89 2,00 2,08

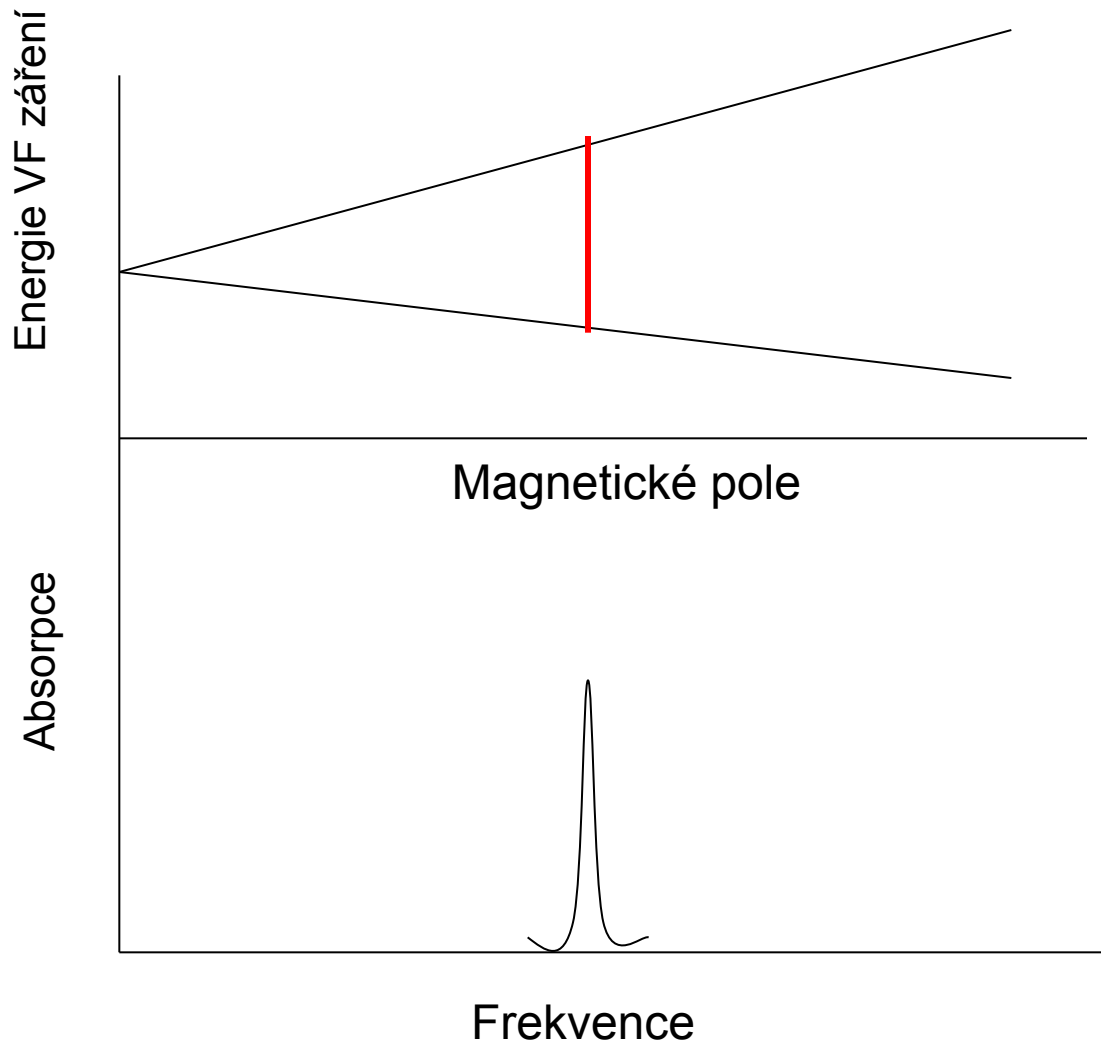
# Nukleární magnetická rezonance

Metoda continuous wave (CW) – konstantní frekvence – mění se  $B_0$



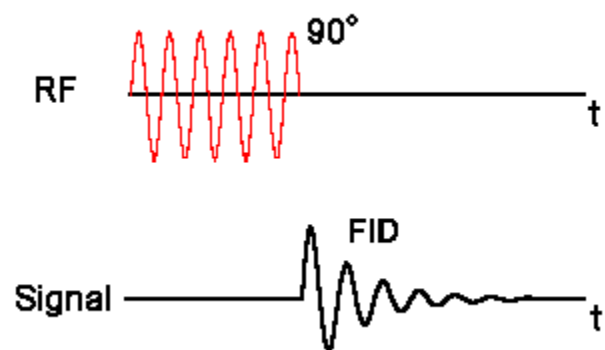
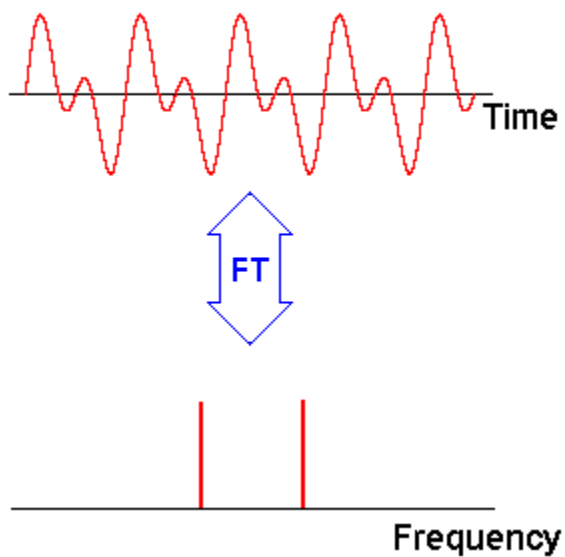
# Nukleární magnetická rezonance

Metoda continuous wave (CW) – konstantní  $B_0$  – mění se frekvence



# Nukleární magnetická rezonance

Fourrierova transformace



# Nukleární magnetická rezonance

Rozpouštědla – Deuterovaná

Acetone  $\text{CD}_3\text{COCD}_3$

Chloroform  $\text{CDCl}_3$

Methylnitrile  $\text{CD}_3\text{CN}$

Water  $\text{D}_2\text{O}$

Diethylether (DEE)  $(\text{CD}_3\text{CD}_2)_2\text{O}$

Dimethyl Sulfoxide (DMSO)  $\text{CD}_3\text{SOCD}_3$

Ethanol  $\text{CD}_3\text{CD}_2\text{OD}$

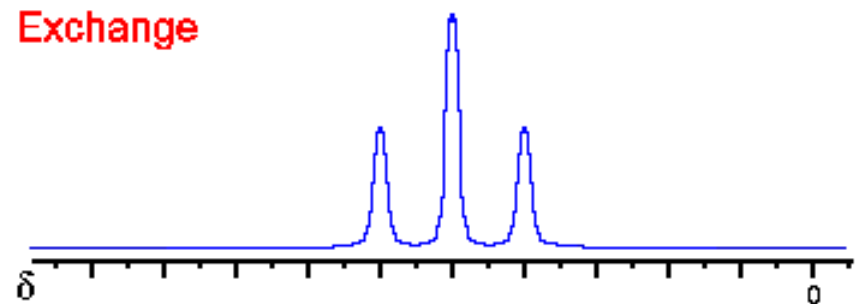
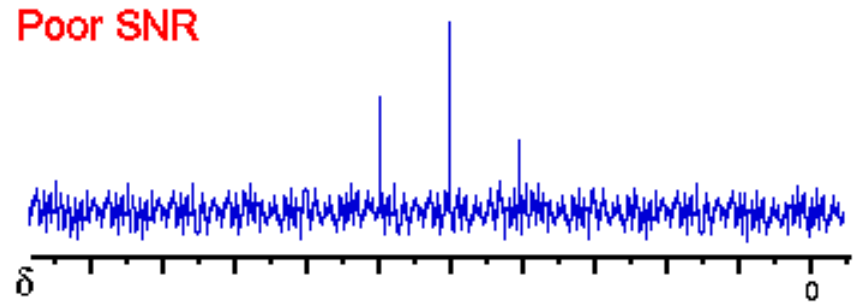
Atd.



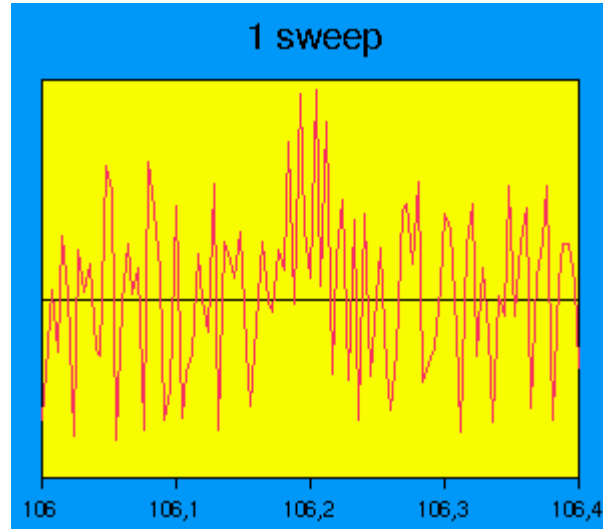
# Nukleární magnetická rezonance

Signal-to noise ratio

Nízká a vysoká koncentrace

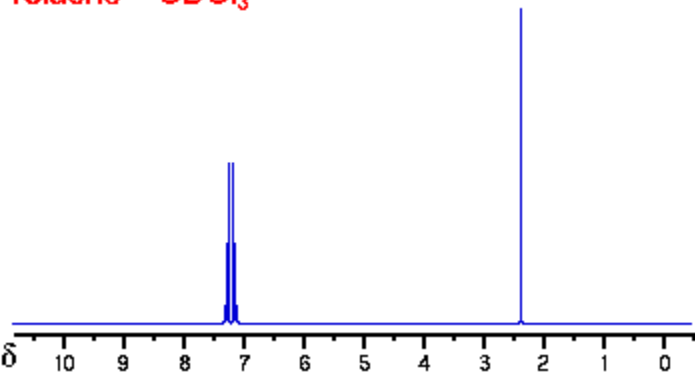


# Nukleární magnetická rezonance

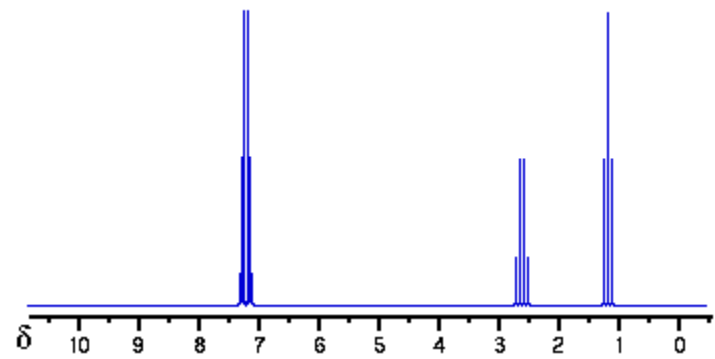


# Nukleární magnetická rezonance

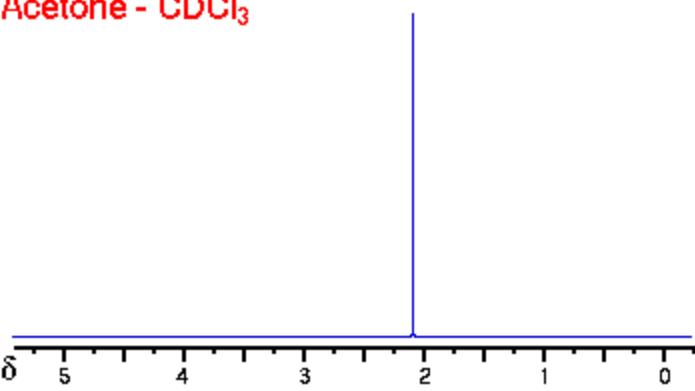
Toluene -  $\text{CDCl}_3$



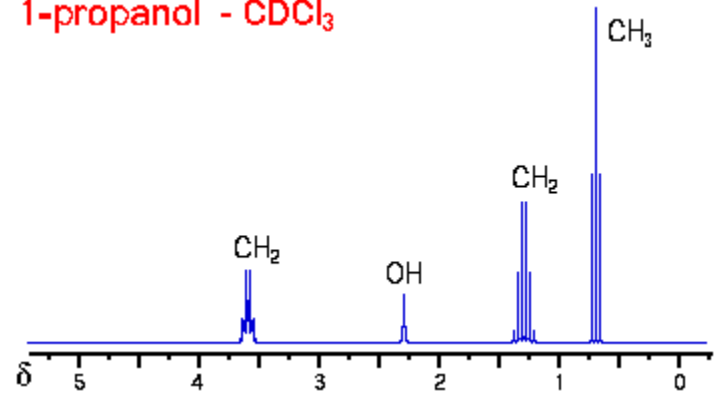
Ethyl benzene -  $\text{CDCl}_3$



Acetone -  $\text{CDCl}_3$

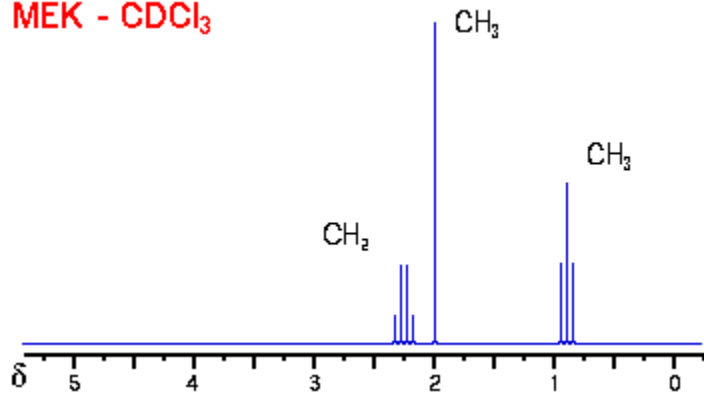


1-propanol -  $\text{CDCl}_3$

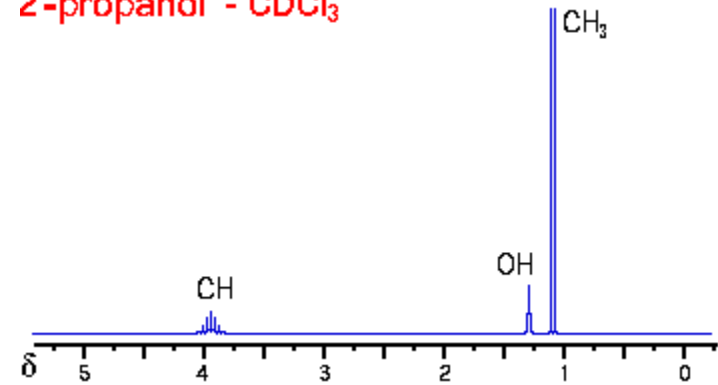


# Nukleární magnetická rezonance

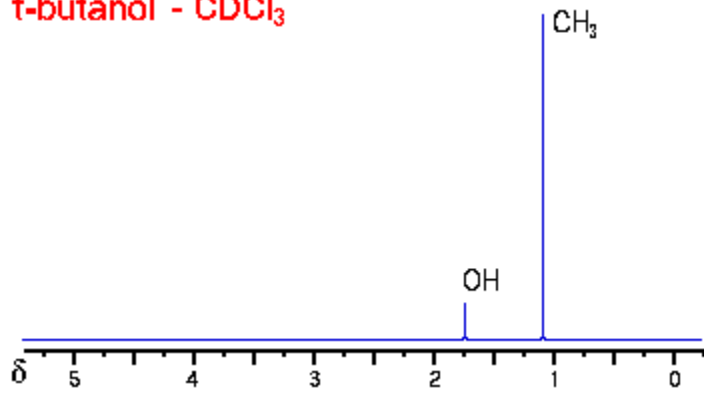
MEK -  $\text{CDCl}_3$



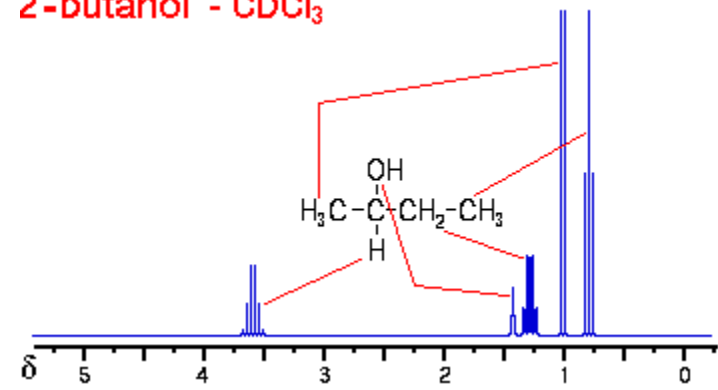
2-propanol -  $\text{CDCl}_3$



t-butanol -  $\text{CDCl}_3$



2-butanol -  $\text{CDCl}_3$



# Nukleární magnetická rezonance- $^{13}\text{C}$

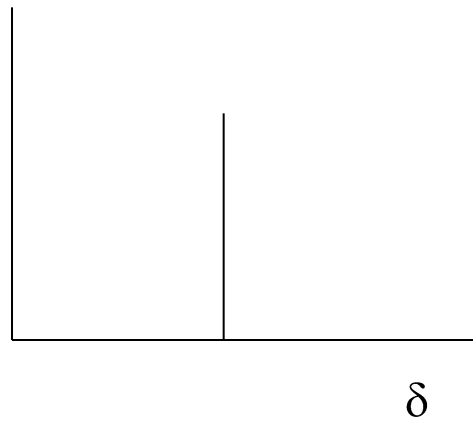
## Carbon-13 Chemical Shifts

Carbon-13* Environment	Chemical Shift Range (ppm)
$\text{CH}_3\text{CH}_2$	0-50
$-\text{C}\equiv\text{C}-$	60-100
$\equiv\text{C}-\text{X}$	70-170
$\text{C}_6\text{H}_6$	65
$\text{CHCl}$ $\text{CHCl}$ (cis)	71
$\text{Ar}$	120-150
$\text{CCl}_4$	97
$\text{COOR}$	170
$\text{COH}$	210
$\text{RCOR}$	220

# Nukleární magnetická rezonance- $^{13}\text{C}$

Spin-spinová interakce mezi  $^{13}\text{C}$ - $^1\text{H}$

Spektrum  $\text{CH}_3\text{I}$



# Nukleární magnetická rezonance-<sup>15</sup>N



## Nitrogen-14 Chemical Shifts \_

<b>Nitrogen-14* Environment</b>	<b>Chemical Shift Range (ppm)</b>
NO <sub>2</sub> Na	-355
NO <sub>3</sub> <sup>-</sup> (aqueous)	-115
N <sub>2</sub> (liquid)	-101
pyridine	-93
bare nucleus	0
CH <sub>3</sub> CN	25
CH <sub>3</sub> CONH <sub>2</sub> (aqueous)	152
NH <sub>4</sub> <sup>+</sup> (aqueous)	245
NH <sub>3</sub> (liquid)	266

# Nukleární magnetická rezonance- $^{31}\text{P}$

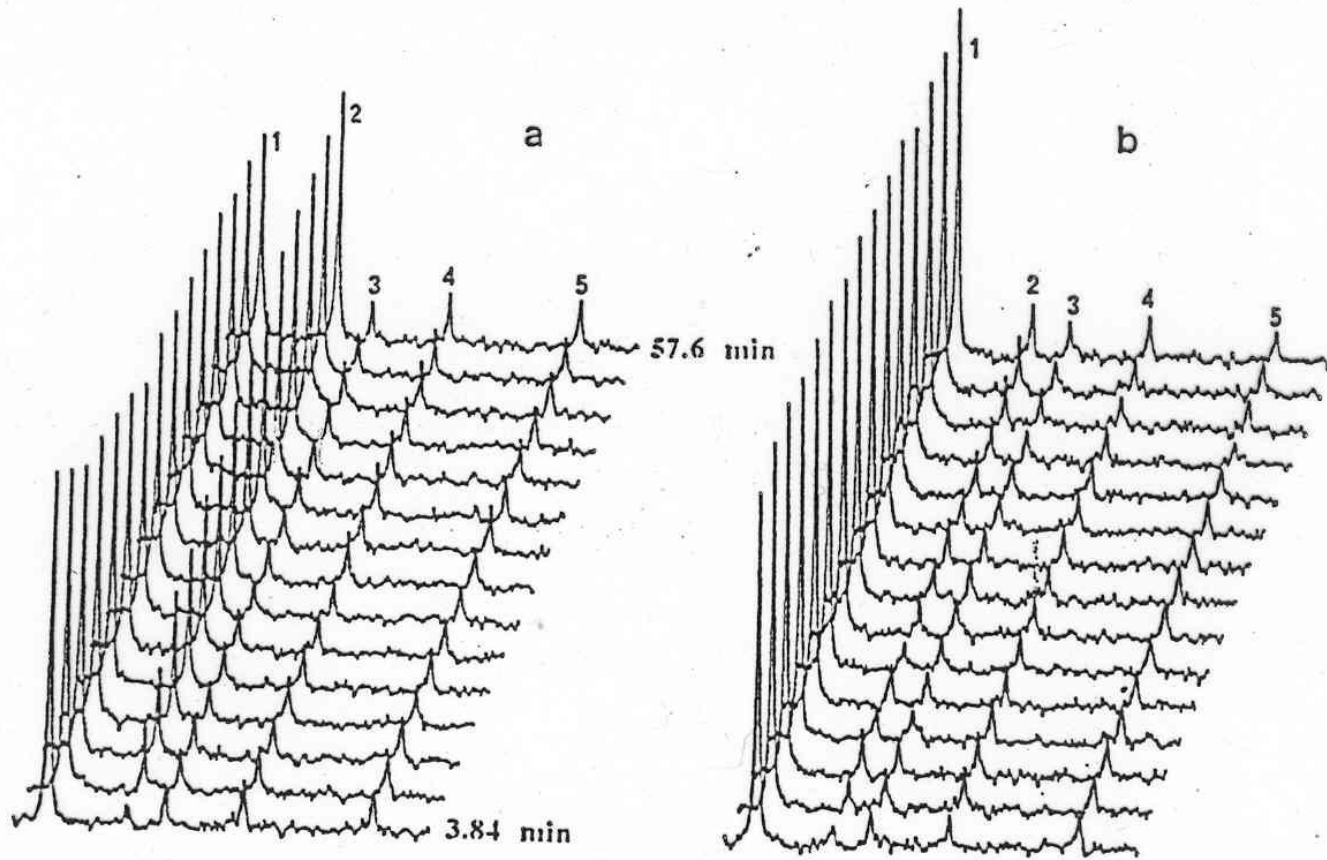


## Phosphorous-31 Chemical Shifts \_

<b>Phosphorous-31 Environment</b>	<b>Chemical Shift Range (ppm)</b>
$\text{PBr}_3$	-228
$(\text{C}_2\text{H}_5\text{O})_3\text{P}$	-137
$\text{PF}_3$	-97
85% phosphoric acid	0
$\text{PCl}_5$	80
$\text{PH}_3$	238
$\text{P}_4$	450



# Nukleární magnetická rezonance- $^{31}\text{P}$



**FIG. 1.** Phosphocreatine synthesis by isolated rat skeletal muscle mitochondria followed by  $^{31}\text{P}$  NMR spectroscopy: two series of typical spectra obtained during one hour with mitochondria (a) in the presence of 1 mM ATP or (b) in the presence of 1 mM ATP and 7 nmole/mg proteins of atractyloside. Peak assignments are (1) Pi, (2) PCr, (3), (4), and (5)  $\gamma$ -,  $\alpha$ -,  $\beta$ - phosphorus of ATP respectively.

# Nukleární magnetická rezonance- $^{31}\text{P}$

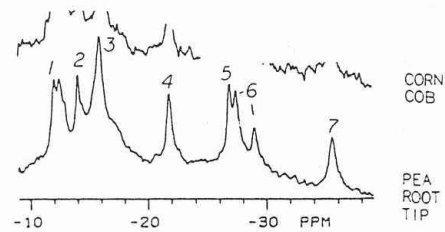


Fig. 12.  $^{31}\text{P}$ -NMR spectra of living plant tissues, under oxygenated conditions. Peak assignments: 1, Glc-6-P; 2, cytoplasmic  $\text{P}_i$ ; 3, vacuolar  $\text{P}_i$ ; 4,  $\gamma\text{ATP}$ ; 5,  $\alpha\text{ATP}$ ; 6, UDPG and nicotinamide adenine nucleotides; 7,  $\beta\text{ATP}$ . [From Roberts (1984), with permission.]

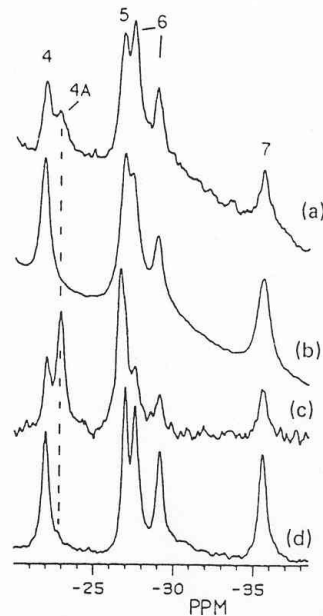


Fig. 19.  $^{31}\text{P}$ -NMR partial spectra of the nucleotide region of (a) hypoxic maize root tips; (b) normoxic maize root tips; (c) extract of normoxic maize root tips, to which commercial ADP has been added; (d) extract of normoxic maize root tips, prior to addition of ADP. Peak assignments as in Fig. 12, except peak 4A,  $\beta\text{ADP}$  (peak 5 will include a contribution from  $\alpha\text{ADP}$ , in addition to  $\alpha\text{ATP}$ ). [From Roberts *et al.* (1985b), with permission.]

# Nukleární magnetická rezonance- $^{31}\text{P}$

Měření intracelulárního pH  
Pomocí posunu signálu  
 $^{31}\text{P}$

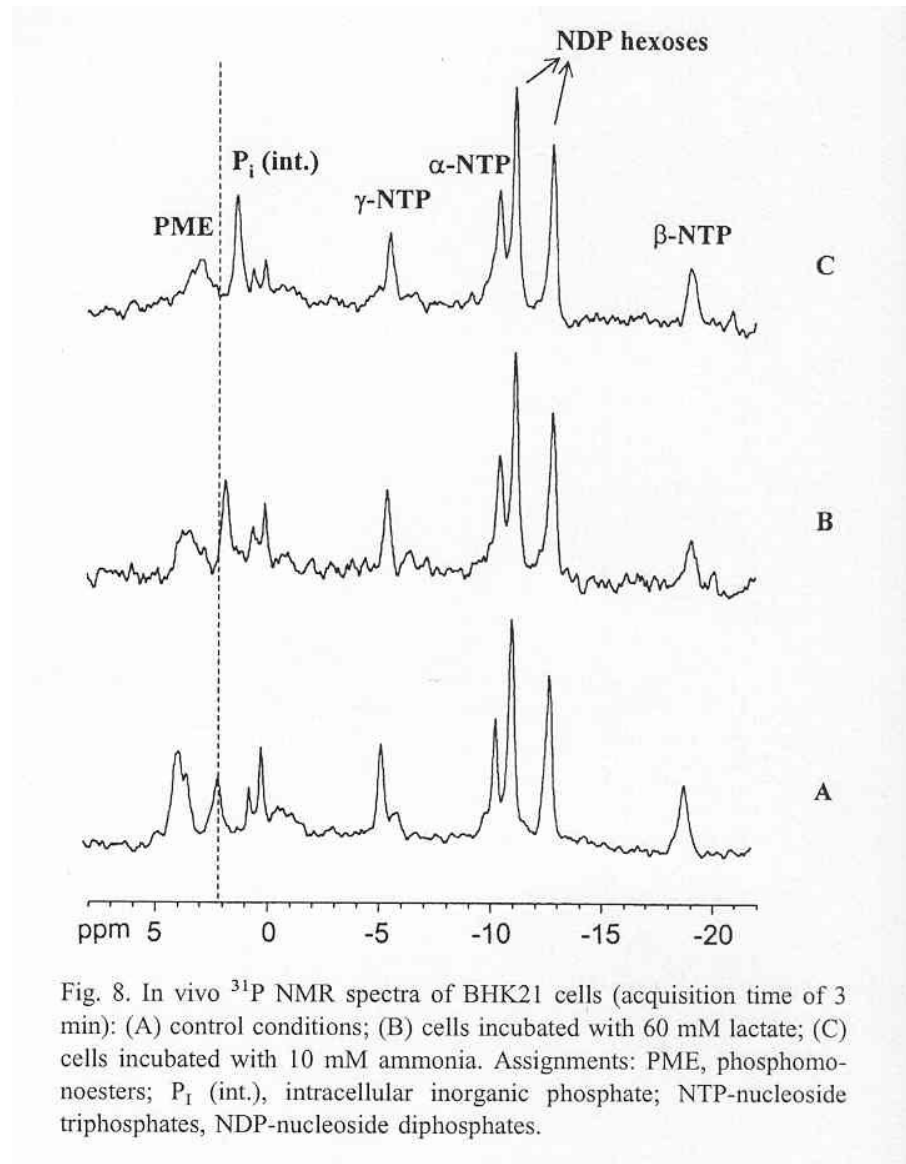


Fig. 8. In vivo  $^{31}\text{P}$  NMR spectra of BHK21 cells (acquisition time of 3 min): (A) control conditions; (B) cells incubated with 60 mM lactate; (C) cells incubated with 10 mM ammonia. Assignments: PME, phosphomonoesters;  $\text{P}_i$  (int.), intracellular inorganic phosphate; NTP-nucleoside triphosphates, NDP-nucleoside diphosphates.

# Nukleární magnetická rezonance- $^{31}\text{P}$

Měření koncentrace nukleotidů  
Pomocí  $^{31}\text{P}$

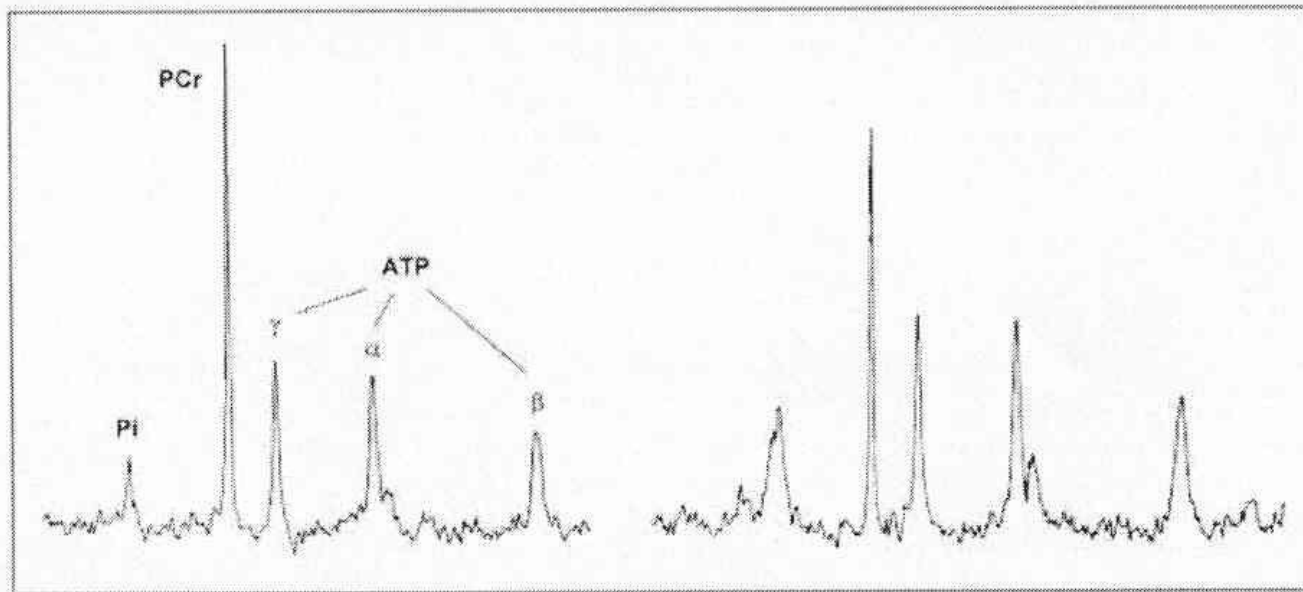


Figure 3 Typical  $^{31}\text{P}$  NMR spectra from an isolated rat heart perfused with glucose only (right) or glucose and pyruvate (left). The major resonances, from left to right, report the relative heart contents of inorganic phosphate (Pi), PCr and the three phosphates of ATP ( $\gamma$ ,  $\alpha$  and  $\beta$ ). Note the increase in PCr resonance area and fall in Pi resonance area when pyruvate is added to the glucose-containing buffer.

# Nukleární magnetická rezonance- $^{13}\text{C}$

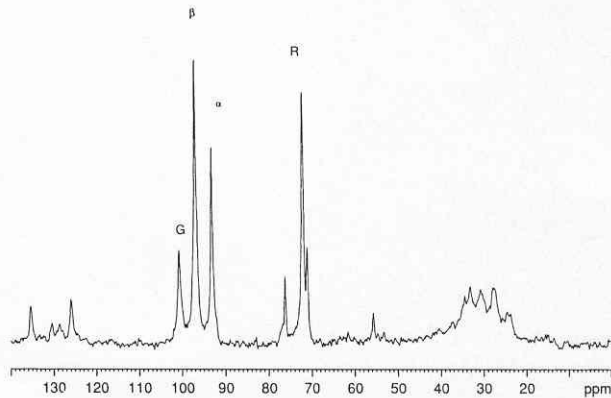


Fig. 7. Representative  $^{13}\text{C}$  NMR spectrum acquired from a perfused rat heart under preischemic conditions after 30 min of labeled perfusion. The abbreviations denote  $[1-^{13}\text{C}]$ glycogen (G), the  $\alpha$ - and  $\beta$ -anomers of  $[1-^{13}\text{C}]$ glucose ( $\alpha$  and  $\beta$ ), and  $[2-^{13}\text{C}]$ ribose (R) used as standard.

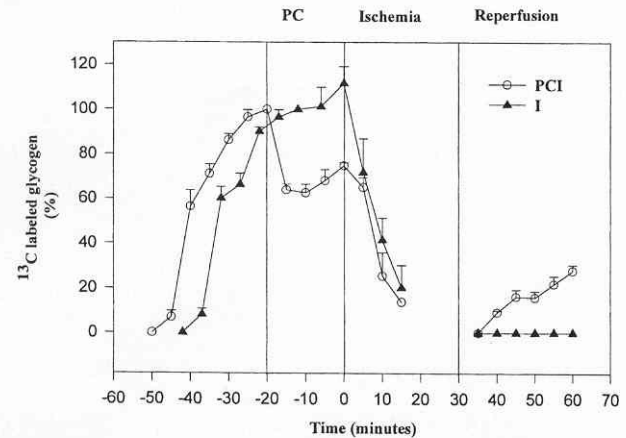


Fig. 8.  $^{13}\text{C}$ -Labeling and mobilization of  $[1-^{13}\text{C}]$ glycogen during protocol set I in preconditioned hearts (PCI,  $n=6$ ,  $\circ$ ) and ischemic controls (I,  $n=6$ ,  $\blacktriangle$ ). The labeled perfusion started at  $t=-50$  min for the preconditioned hearts and  $t=-42$  min for the controls. During the first 30 min of perfusion (stabilization), the behavior of the two groups was almost identical.  $[1-^{13}\text{C}]$ -Glycogen was partially reduced by the first cycle of preconditioning ischemia and was not further reduced by subsequent cycles. In the PCI group, preischemic glycogen proved to be lower than that in Group I, with reduced  $[1-^{13}\text{C}]$ glycogen mobilization during early ischemia ( $t=0-15$  min). Only the PCI group resumed glycogen synthesis. Mean  $\pm$  S.E.M.

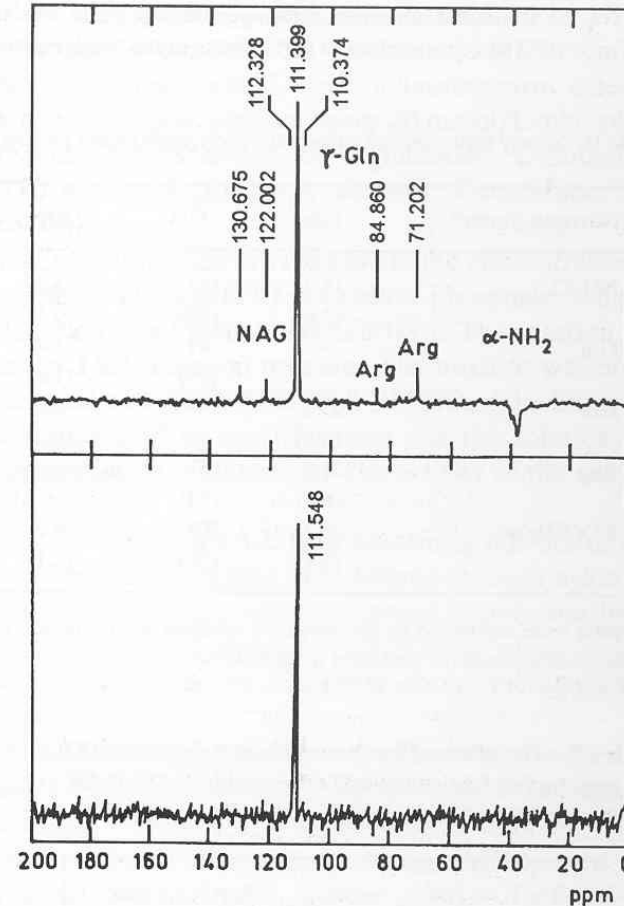
# Nukleární magnetická rezonance – $^{15}\text{N}$

Asimilace amoniaku sledovaná pomocí  $^{15}\text{N}$

Hlíva ústříčná

Extrakt mycelia narostlého na  $^{15}\text{NH}_4\text{Cl}$

Mycelium 1 hod. po přidání  $^{15}\text{NH}_4\text{Cl}$



**Fig. 1.**  $^{15}\text{N}$ -NMR spectrum (ppm) of cell-free extract from the mycelium of *P. ostreatus*. *Top*: mycelium grown in  $^{15}\text{NH}_4\text{Cl}$ , *bottom*: mycelium 1 h after the addition of  $^{15}\text{NH}_4\text{Cl}$ .

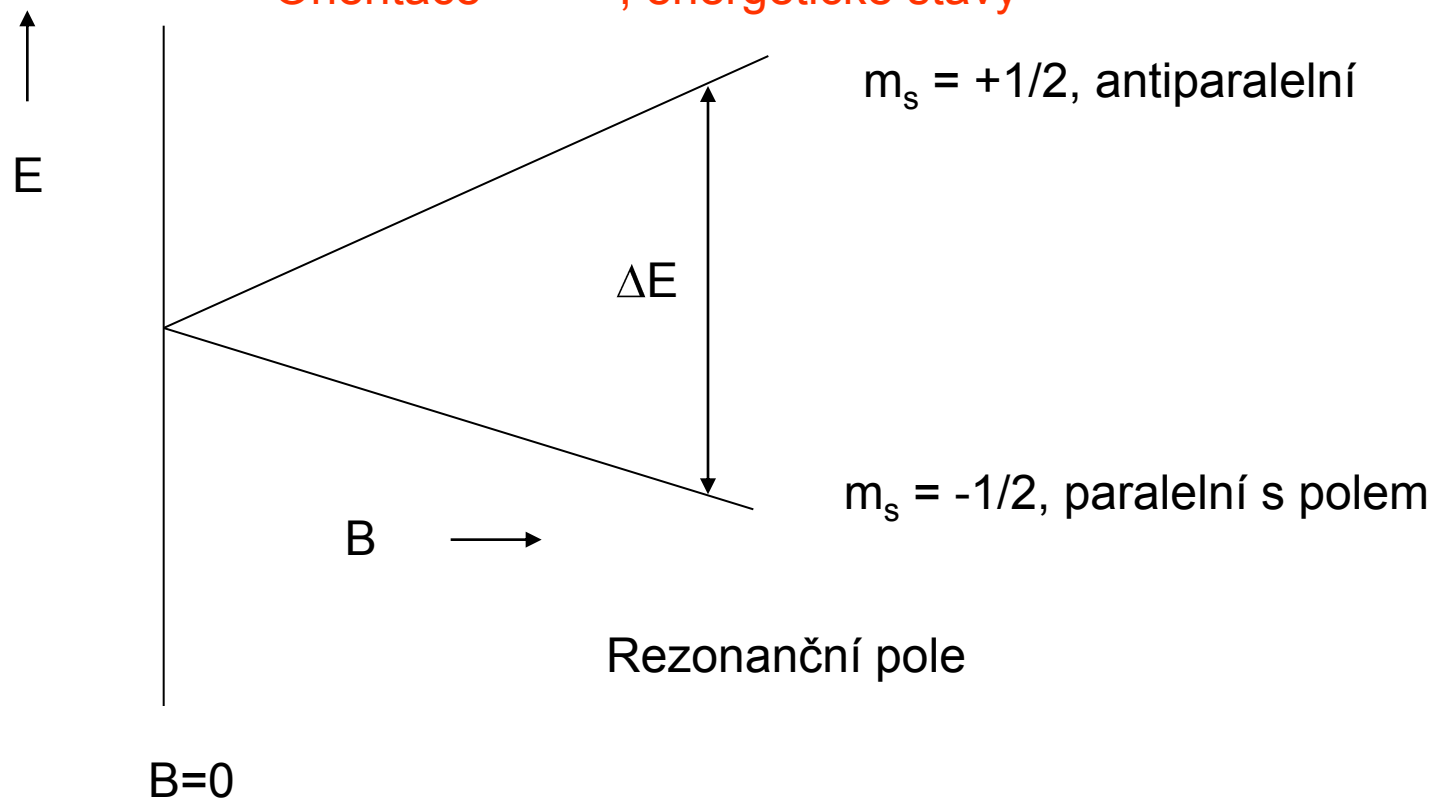
# EPR - ESR

- Zde výtah pro základní pochopení
- Pro zájemce o bližší seznámení

<http://cheminfo.chemi.muni.cz/ianua/epr/index.htm>

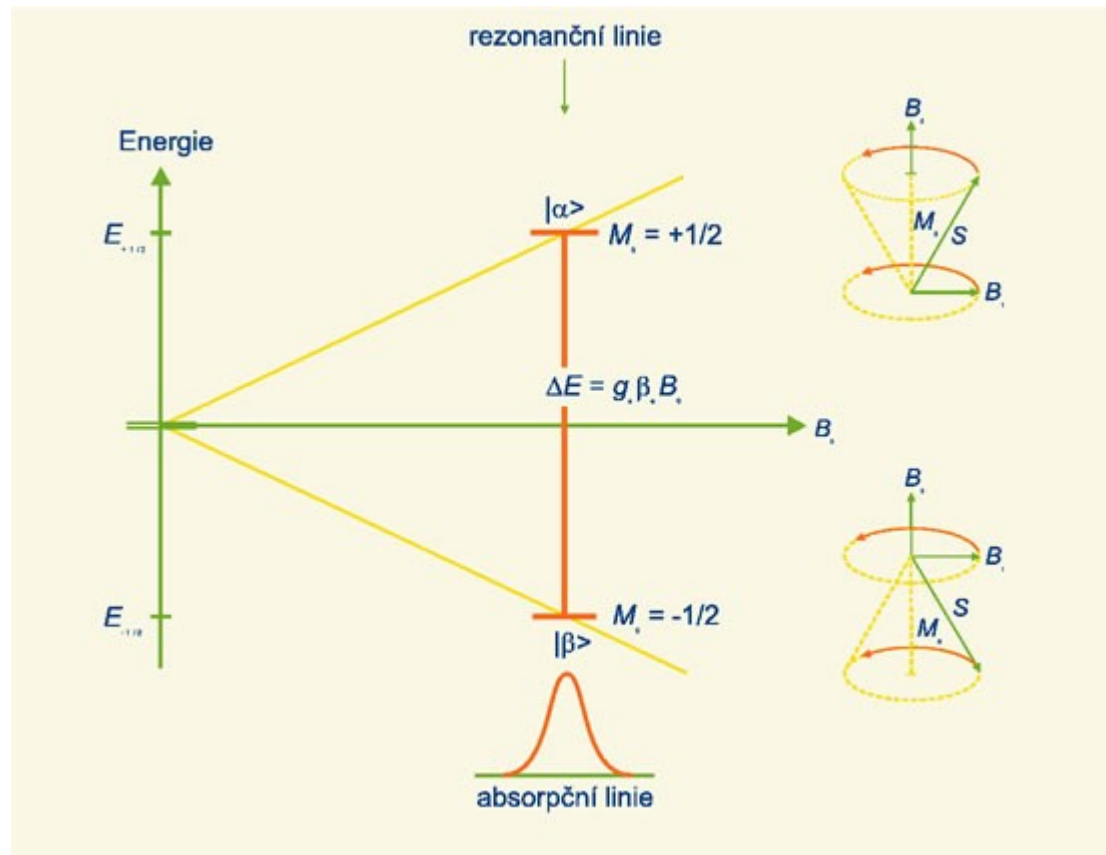
# EPR – elektronová spinová rezonance

Nepárový elektron – nenulový spin –  $|S| = 1/2$   
Orientace – + - , energetické stavy





# EPR – elektronová spinová rezonance



# EPR – elektronová spinová rezonance

$$\Delta E = h\nu = g\mu B_0$$

Počet jader v jednotlivých orientacích  
Intenzita signálu

$$N_1/N_2 = e^{-\Delta E/kT}$$

$\Delta E$  rozdíl energií

$h$  – Planckova konstanta

$\nu$  – frekvence mikrovln

$g$  – Landéův faktor  
spin-orbitální interakce

$\mu$  – Bohrov magneton

$B_0$  – magnetická indukce

$$B_0 = 0,1 - 0,5 \text{ T}; \quad \nu = 10 - 100 \text{ GHz}$$

# EPR – elektronová spinová rezonance

- Absorpce fotonů – MW oblast

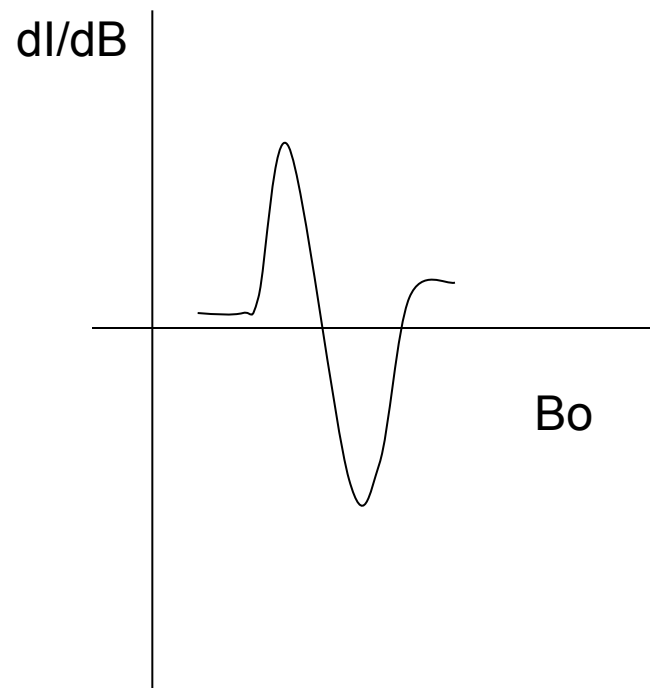
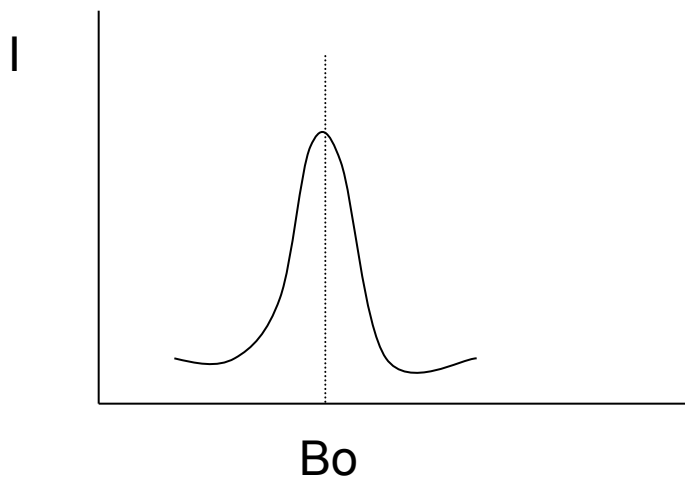
$$\nu = f(B_0)$$

změna  $\nu$  nebo  $B_0$  – častější

spektrum základní

derivace – standartně

# EPR – elektronová spinová rezonance



# EPR – elektronová spinová rezonance

Interakce s protony – štěpení píků

1 proton  $I = \frac{1}{2}$

multiplicita =  $n$  (protonů ev. 2nI jádra) + 1, intenzita píků  $(x + 1)^n$   
platí pro ekvivalentní jádra (jinak složitější)

$n = 1$  – 1:1

$n = 2$  – 1:2:1

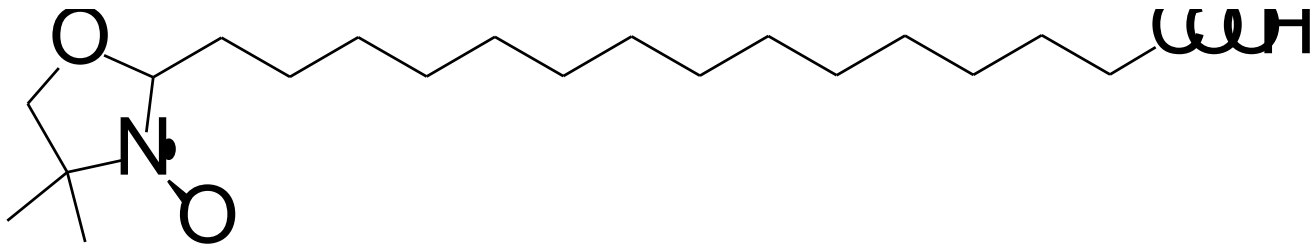
$n = 3$  – 1:3:3:1



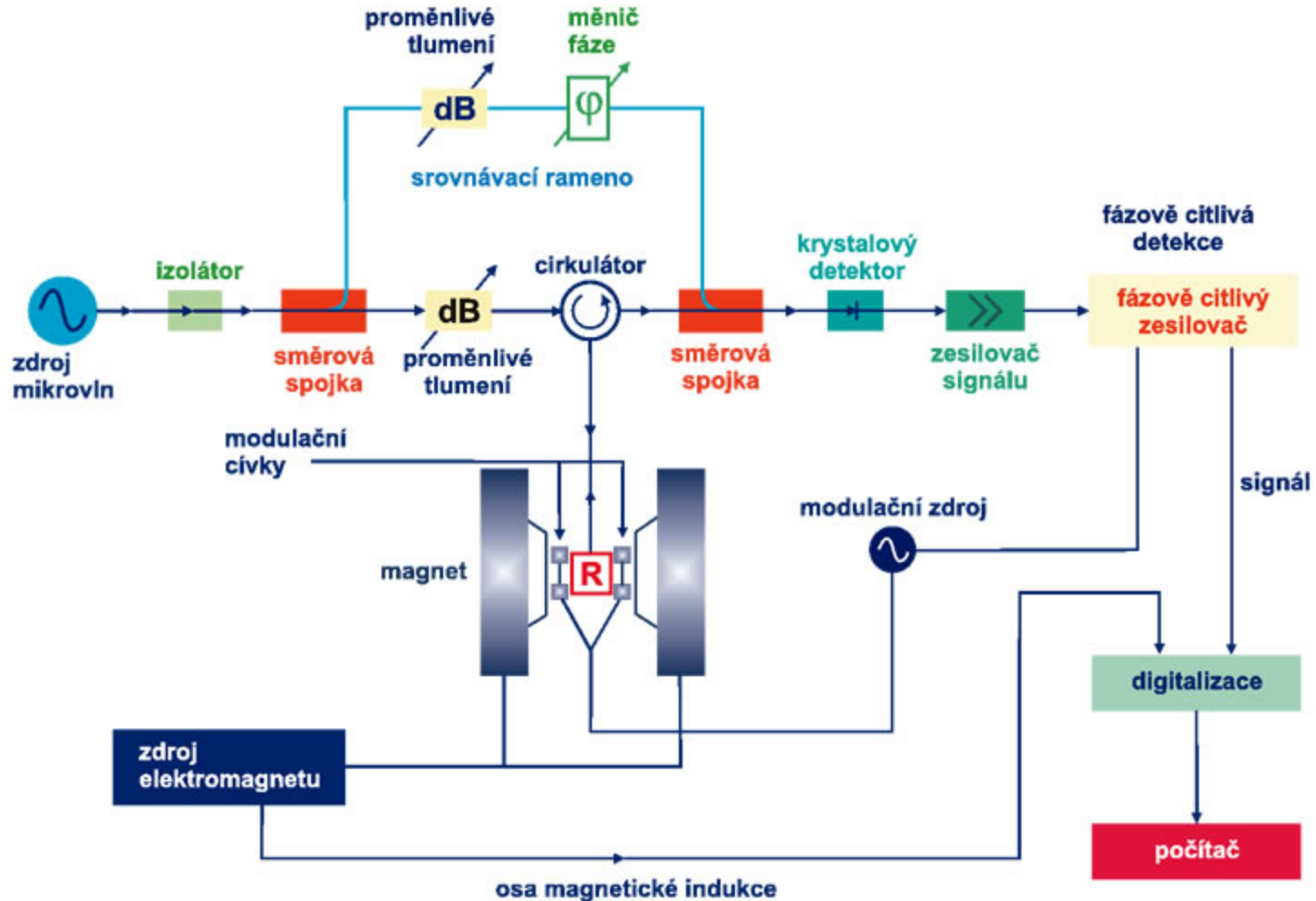
# EPR – elektronová spinová rezonance

Využití v biochemii

- Studium radikálů (peroxydy)
- Studium konformace – spinové značky – stabilní radikály
- Studium hemu

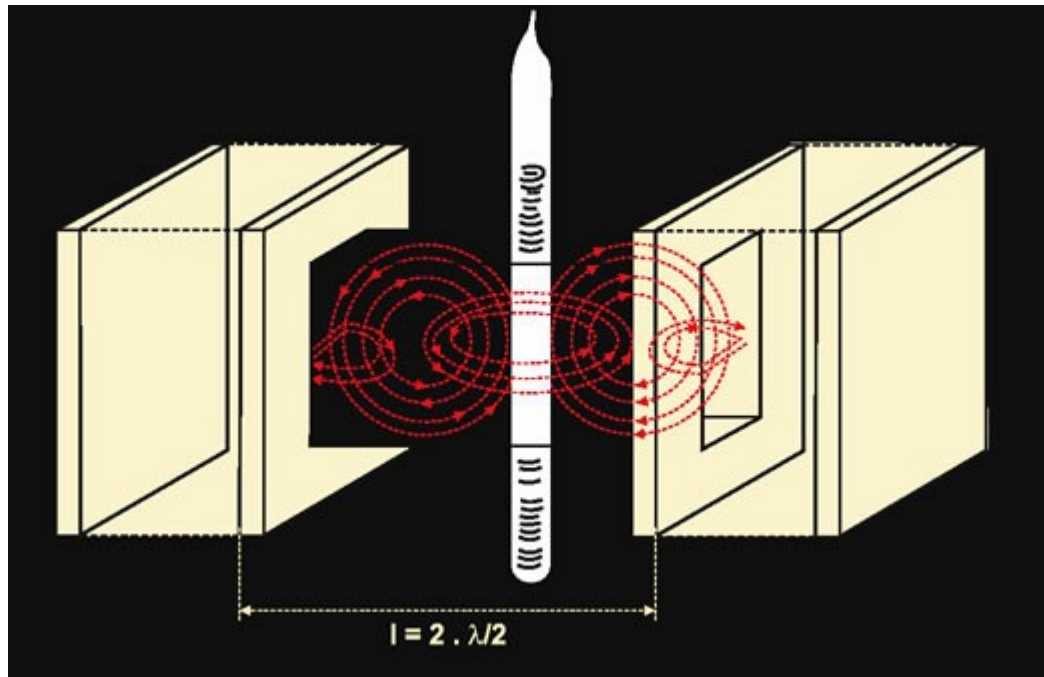


# EPR - schema

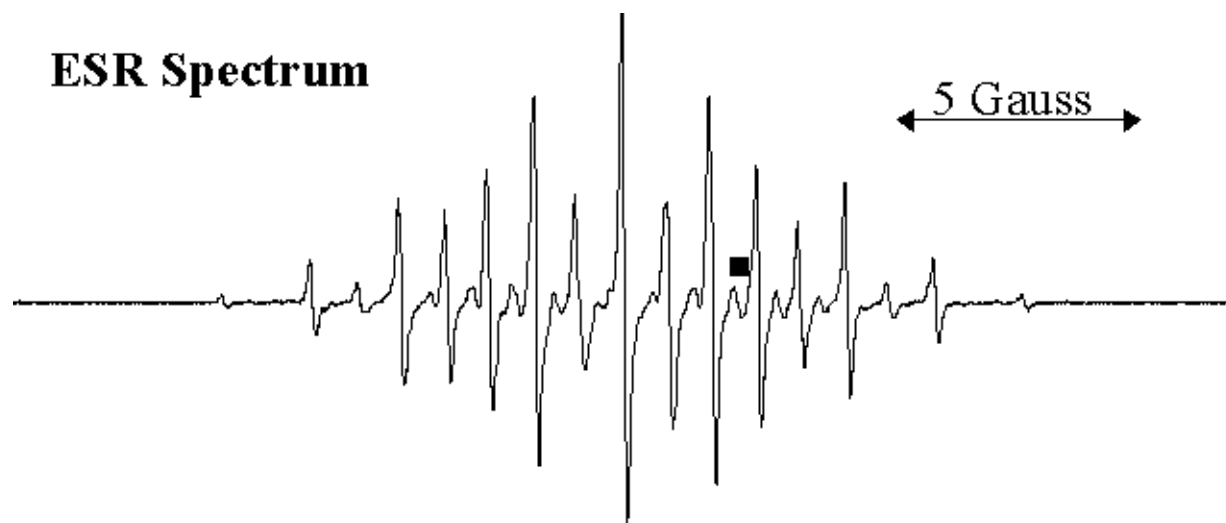
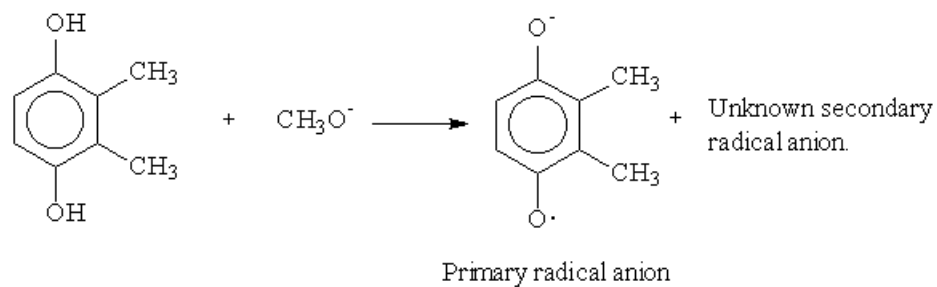




# EPR - měření



# EPR – elektronová spinová rezonance



# EPR – elektronová spinová rezonance

EPR spektrum NADH-UQ reductasy  
signál UQ  
Signál 2Fe2S potlačen,  
Signál FMN radikálu interferuje

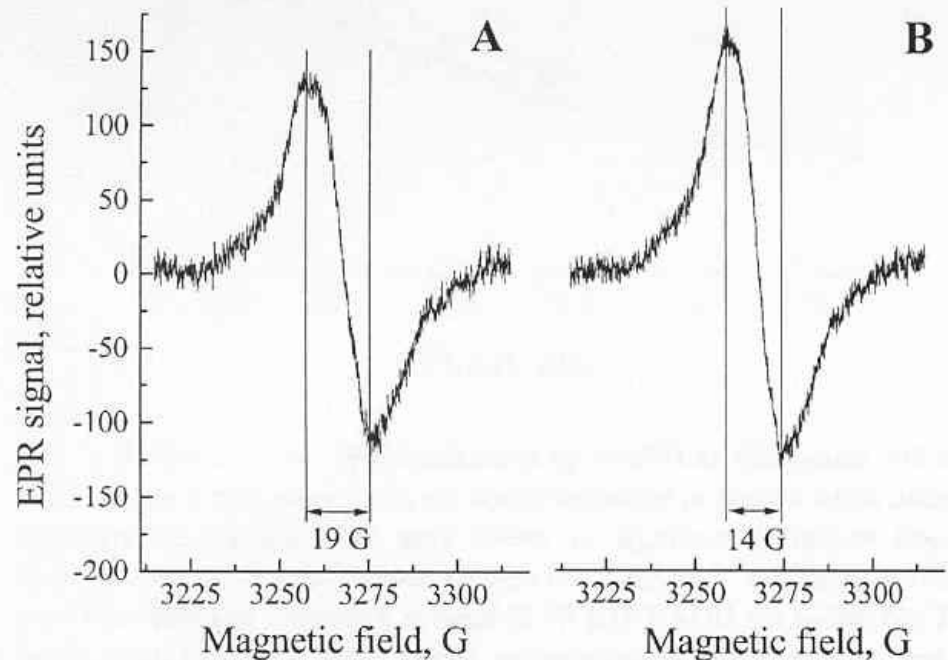


Fig. 1. EPR spectra of the radical signals in Na<sup>+</sup>-NQR from *V. harveyi*. (A) Air-oxidized, (B) dithionite-reduced. Sample conditions: 50 mM HEPES/Tris (pH 7.5), 200 mM NaCl and 0.05% DM, protein concentration 3 mg ml<sup>-1</sup>, (B) dithionite, 5 mM. EPR conditions: microwave frequency, 9.15 GHz; microwave power, 100 μW; temperature, -40 °C; modulation amplitude, 0.2 mT.

# EPR – elektronová spinová rezonance

EPR spektrum ferredoxinu  
z mořské řasy  
(3Fe-4S) $1+ / 0$   
Typický signál

M.M. Pereira et al. / *Biochimica et Biophysica Acta* 1601 (2002) 1–8

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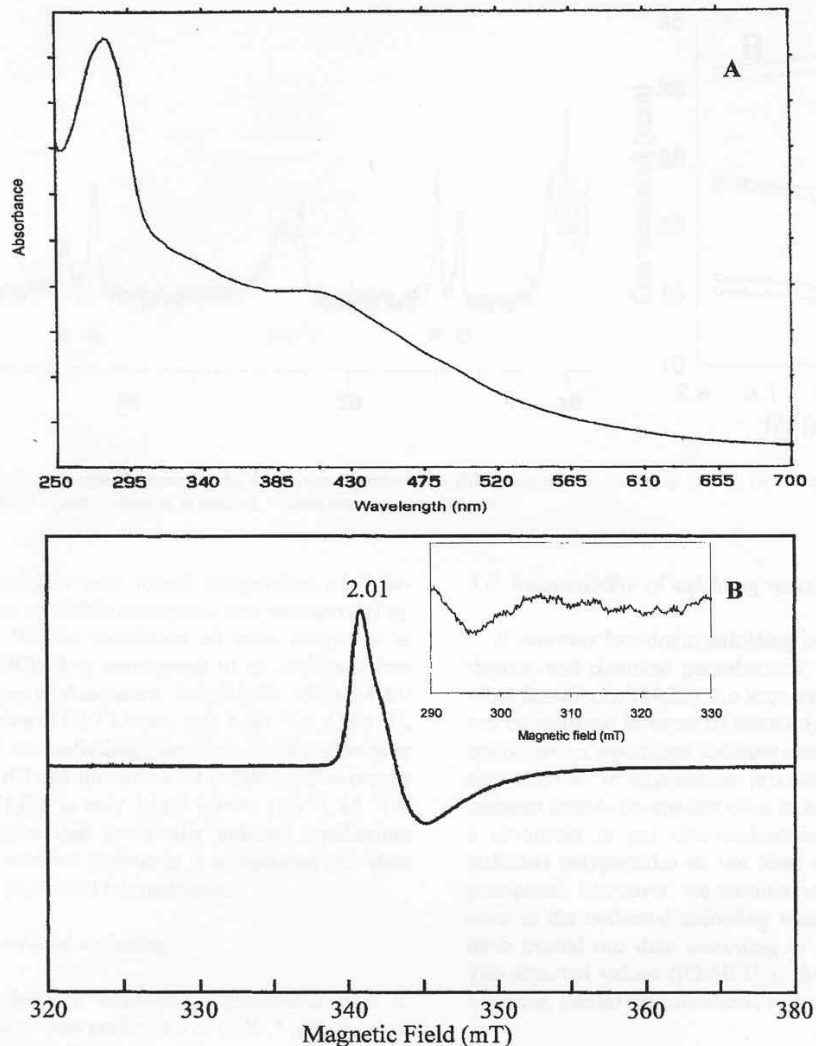


Fig. 2. (A) UV-visible spectrum of the oxidised ferredoxin from *R. marinus*. (B) EPR spectra of the ferredoxin from *R. marinus* in oxidised form and perpendicular mode (main spectrum) and reduced form and parallel mode (insertion). EPR conditions—temperature: 10 K; microwave frequency: 9.64 GHz (perpendicular mode) and 9.43 GHz (parallel mode); microwave power: 2.4 mW; modulation amplitude: 0.9 mT. Protein concentration 50  $\mu$ M (A) and 100  $\mu$ M (B).

# EPR – elektronová spinová rezonance

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EPR spektrum cyt bc<sub>1</sub> – mutant  
M183K, M183H

Fe<sup>3+</sup> hem (3,78 = cyt bL 3,44, cyt  
bH,  
2,94 bis-His cyt b nebo c<sub>1</sub>)

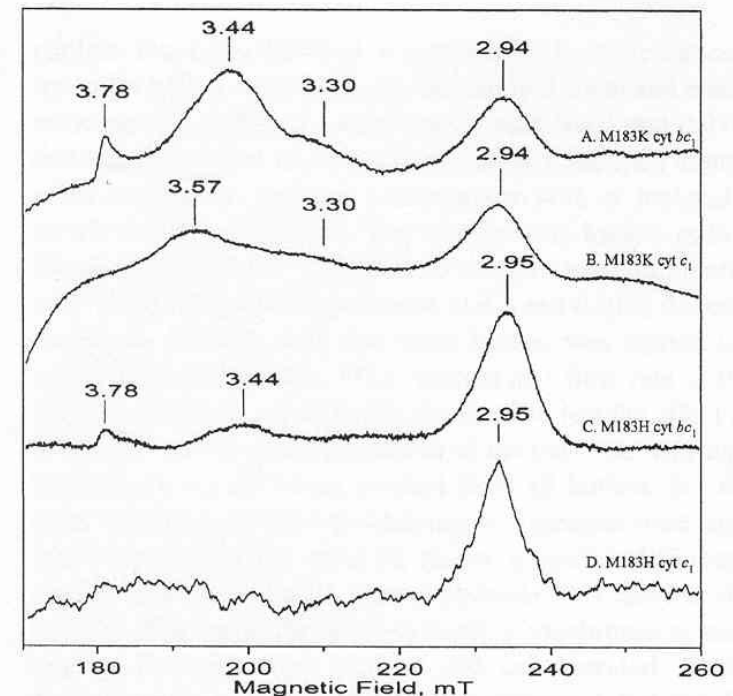


Fig. 2. X-band EPR spectra of *Rb. capsulatus* cytochromes. (A) Cytochrome *bc*<sub>1</sub> complex containing M183K cytochrome *c*<sub>1</sub> (39  $\mu$ M). (B) Purified M183K cytochrome *c*<sub>1</sub> (148  $\mu$ M). (C) Cytochrome *bc*<sub>1</sub> complex containing M183H cytochrome *c*<sub>1</sub> (29  $\mu$ M). (D) Purified M183H cytochrome *c*<sub>1</sub> (24  $\mu$ M). The buffer conditions are: 100 mM Tris–DCl (pD=8.0), 10 mM NaCl, 0.1% dodecyl maltoside and 55% *d*<sub>3</sub>-glycerol for (A) and (C); 25 mM Tris–DCl (pD 8.0), 10 mM NaCl, 0.25% cholate and 55% *d*<sub>2</sub>-ethylene glycol for (B); 25 mM Tris–DCl (pD 8.0), 10 mM NaCl, 1% cholate and 55% *d*<sub>3</sub>-glycerol for (D). All spectra were recorded at 10 K and instrument settings were: number of scans, 5; microwave power, 10 mW; modulation amplitude, 1.0 mT; microwave frequency, 9.59 GHz.

# EPR – elektronová spinová rezonance

EPR spektrum oxidace radikálů  
LDL vyvolaných H<sub>2</sub>O<sub>2</sub>

Spektrum radikálu tokoferolu (A)

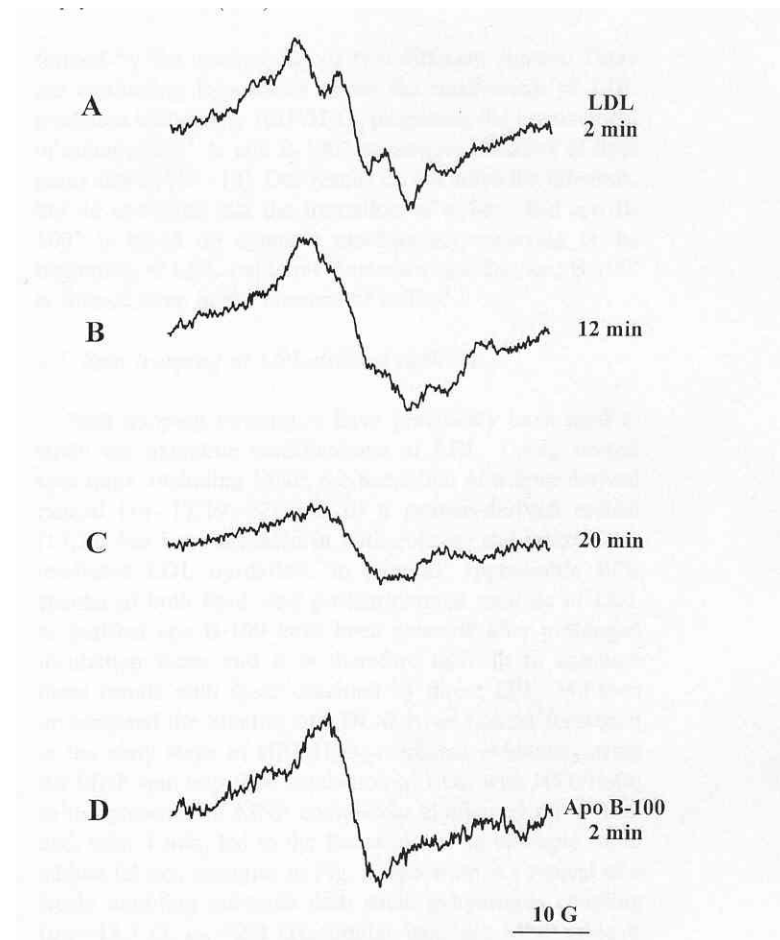


Fig. 1. EPR spectra at 37 °C of LDL and purified apo B-100 treated with HRP/H<sub>2</sub>O<sub>2</sub>. (A) Spectrum of LDL (1.5 mg/ml) recorded after 2 min of incubation with HRP (10 μM) and H<sub>2</sub>O<sub>2</sub> (2.5 mM) in phosphate-buffered saline, DTPA (0.1 mM), pH 7.4; (B) sample (A) after 12 min of incubation, and (C) sample (A) after 20 min of incubation. (D) Spectrum of apo B-100 (4 mg/ml) recorded after 2 min of incubation with HRP (10 μM) and H<sub>2</sub>O<sub>2</sub> (2.5 mM), in 10 mM Tris-HCl and 10 mM sodium deoxycholate, DTPA (0.1 mM), pH 9.0. Spectrometer conditions were as follows: modulation amplitude, 5 G; time constant, 82 ms; sweep time, 21 s; number of scans, 20.