

6. Nuclear Magnetic Resonance (NMR) Spectroscopy.

In this chapter we will discuss some basic principles of NMR spectroscopy and some very basic applications. A comprehensive treatment of NMR spectroscopy would require a separate series of lectures in 1 or even 2 semesters. This introduction will make beginners familiar with the ideas behind the NMR technique and the application of the method to smaller molecules.

6.1. The Magnetic Resonance Experiment.

6.1.1 Spin and magnetic moment of nuclei in an applied magnetic field.

As we have already pointed out for previously discussed techniques, the interaction of electromagnetic radiation with matter requires a change in the dipole moment of the molecule that interacts with the radiation. In optical and infrared spectroscopy, a change of the electrical dipole moment in the molecule was caused by the electrical field vector of the radiation. Magnetic resonance phenomena are interactions between the magnetic field vector of the electromagnetic radiation and the magnetic dipole of the molecule. Since the magnetic and the electric field vector are in phase, the frequency of the change in the magnetic field vector is the same as the frequency of the electrical field vector (just the frequency of the electromagnetic radiation). Absorption occurs if the energy difference between the magnetic dipole moment of the molecule in the ground state and the magnetic dipole moment in the excited state is equal to $h\nu$.

The magnetic resonance phenomenon is based on the fact that elementary particles have a property called “spin”, which is often seen as a rotation of the particle around its axis. It was shown by quantum theory that for example electrons have two spin states. This was experimentally first observed in absorption spectra of sodium, where the so called sodium D absorption line has a doublet fine structure (the line is split because of the angular momentum of the electron in the excited state (3p), that induces a magnetic field towards which the magnetic field induced by the spin can be parallel or antiparallel. Thus absorption takes place to different energy levels, depending on the spin of the electron in the excited state (in the electronic ground state 3s, the electron does not have an angular momentum).

In nuclear magnetic resonance the spin of the nucleus of an atom induces a magnetic dipole moment. Spin and magnetic dipole moment are proportional

$$\vec{\mu}_I = g_N \gamma_N \vec{I} \quad \text{Eq. 6.1}$$

The constant

$$\gamma_N = \frac{q}{2m} \quad \text{Eq. 6.2}$$

is termed the magnetogyric ratio of the particle.

The constant g_N is specific for a nucleus. For example, a free proton has the value $g_N=5.585$. In the presence of an external magnetic field, the nucleus of interest can be in to different energetic states that are given by *the dipole moment of the nucleus and by the magnitude of the external magnetic field*. The energy of the nucleus is then

$$E = \vec{\mu} \cdot \vec{B}_0 = g_N \gamma_N \vec{I} \cdot \vec{B}_0 \quad \text{Eq. 6.3}$$

Quantum theory shows that the spin can be conveniently expressed by a spin quantum number I , that gives rise to an angular momentum. This angular momentum is of the magnitude:

$$\vec{I} = \sqrt{I(I+1)}\hbar \quad \text{Eq. 6.4}$$

Nucleus	Mass	Charge	Spin	Natural abundance (%)	Relative sensitivity
^1H	1	1	1/2	99.98	1.000
^2H	2	1	1	0.016	0.0096
^{12}C	12	6	0	98.96	-
^{13}C	13	6	1/2	1.11	0.016
^{14}N	14	7	1	99.64	0.001
^{15}N	15	7	1/2		
^{16}O	16	8	0		-
^{17}O	17	8	5/2		
^{19}F	19	9	1/2	100	0.834
^{31}P	31	15	1/2	100	0.066
^{32}S	32	16	0	95.06	-

Table 1 The nuclear spin originates from the protons and the neutrons. Nuclei with odd mass and even charge number often have no spin, while nuclei with even mass and odd charge number often have a spin of 1/2 or an odd multiple of 1/2.

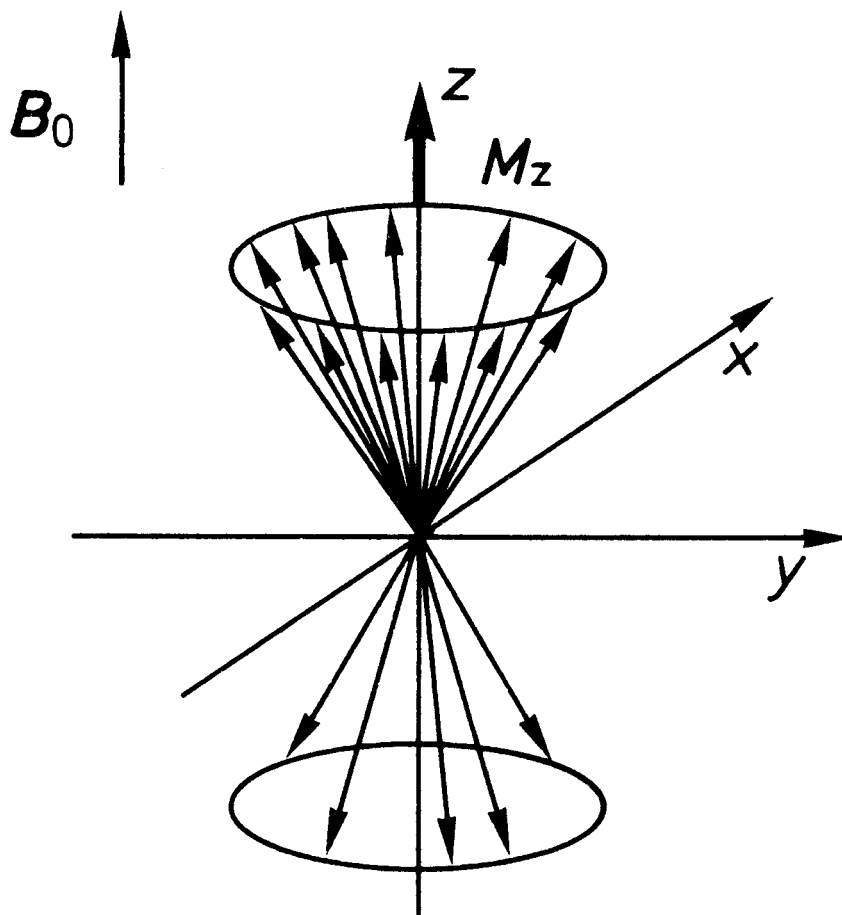
The spin of a charge particle gives rise to a magnetic dipole moment:

$$\vec{\mu}_I = g_N \gamma_N \vec{I} = g_N \gamma_N \sqrt{I(I+1)}\hbar = g_N \beta_N \sqrt{I(I+1)} \quad \text{Eq. 6.5}$$

The constant $\beta_N = \hbar$ is the so called Bohr Magneton of the nucleus.

The spin quantum number I is specific for the particle of interest. It can be only *one* value (depending on the particle) out of the set $0, 1/2, 1, 3/2, 2, 5/2, \dots$ (Table 1).

In a magnetic field, the spins of the charged particles precess around the z -axis that is defined by the direction of the magnetic field (Figure 6.1). The frequency by which the spins precess



Precession of a group of identical nuclei in a magnetic field. The field causes a macroscopic magnetization in the direction of the applied magnetic field, which defines the z -direction. In x and y direction, there is a statistical distribution of the nuclei and thus no macroscopic magnetization.

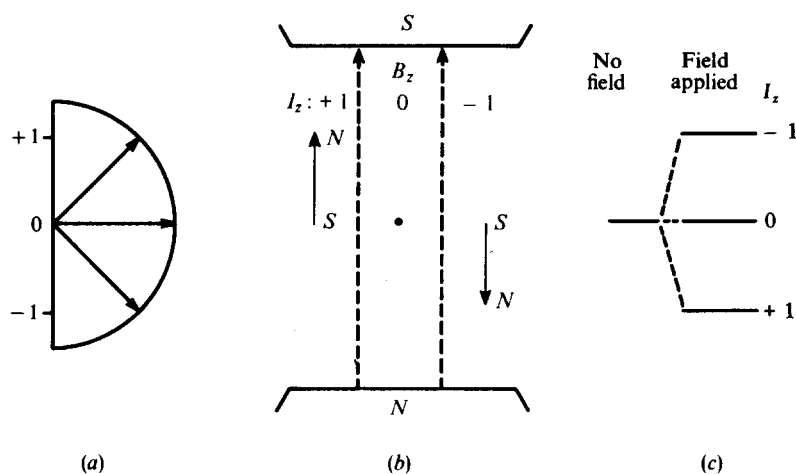
Figure 6.1 Precession of spinning nuclei around the direction of a magnetic field B_0 .

around the magnetic field vector is called Larmor frequency and given by

$$\omega = \frac{\text{magnetic moment}}{\text{angular momentum}} B_0 = \frac{\mu B_0}{2\pi I} = \frac{g\beta_N}{h} B_0, \text{ (expressed in Hz)}$$

Eq. 6.6

The directions that the spin can take in a magnetic field depend on the spin quantum number of the nucleus (Figure 6.2).



A nucleus with spin quantum number $I=1$ can have three orientations (a). In a magnetic field the magnetic dipole of the spinning nucleus will orient itself in three possible directions (b) that correspond to three energetic states of the nucleus (c).

Figure 6.2 Possible orientations of the magnetic dipole moments of precessing nuclei with spin quantum number $I=1$ with respect to a magnetic field.

Nuclei with a quantum number of $I=1$ can have components in the direction of the magnetic field that are $m_I=-1$, $m_I=0$, and $m_I=+1$. In general, nuclei with an integral spin quantum number I can have the z -components

$$m_I = I, I-1, \dots, 0, \dots, -(I-1), -I ; \text{ for } I \text{ integral}$$

while nuclei with a spin quantum number that is a half-integral number can have the z -components

$$m_I = I, I-1, \dots, 1/2, -1/2, \dots, -I ; \text{ for } I \text{ as an odd multiple of } 1/2.$$

which gives $2I+1$ components in each case. These components are degenerate in absence of a magnetic field, but split into different energy levels if a magnetic field is present. The component of the magnetic dipole moment of the precessing spins that is parallel to the

magnetic field B_0
is then given by:

$$\mu_z = g_N \beta_N m_I \quad \text{Eq. 6.7}$$

For a nucleus with a spin quantum number $I=1$, the angular momentum is given by

$$|I| = \sqrt{I(I+1)}\hbar = \sqrt{2}\hbar \quad \text{Eq. 6.8}$$

with a corresponding magnetic moment of

$$\mu_I = g_N \beta_N |I| = g_N \beta_N \sqrt{I(I+1)} = g_N \beta_N \sqrt{2}. \quad \text{Eq. 6.9}$$

The vector component in z-direction of such a spin will take the values of $m_I = -1, 0$, or $+1$ (from quantum mechanics). The orientation of the spin that corresponds to $m_I = -1$ has a magnetic dipole that is opposed to the applied external magnetic field B_0 . Thus, this state corresponds to a higher energy level than the magnetic dipole of the $I=+1$ resonance line.

For a nucleus with $I=1/2$ we obtain in analogy $m_I = -1/2, +1/2$ and an angular momentum of the spinning nucleus is given by

$$|I| = \sqrt{I(I+1)} = \sqrt{\frac{1}{2}\left(1 + \frac{1}{2}\right)} = \frac{1}{2}\sqrt{3}, \quad \text{Eq. 6.10}$$

which corresponds to a magnetic momentum of

$$\mu_I = g_N \beta_N |I| = g_N \beta_N \sqrt{I(I+1)} = g_N \beta_N \sqrt{3}/2 \quad \text{Eq. 6.11}$$

with a z-component in an external magnetic field of

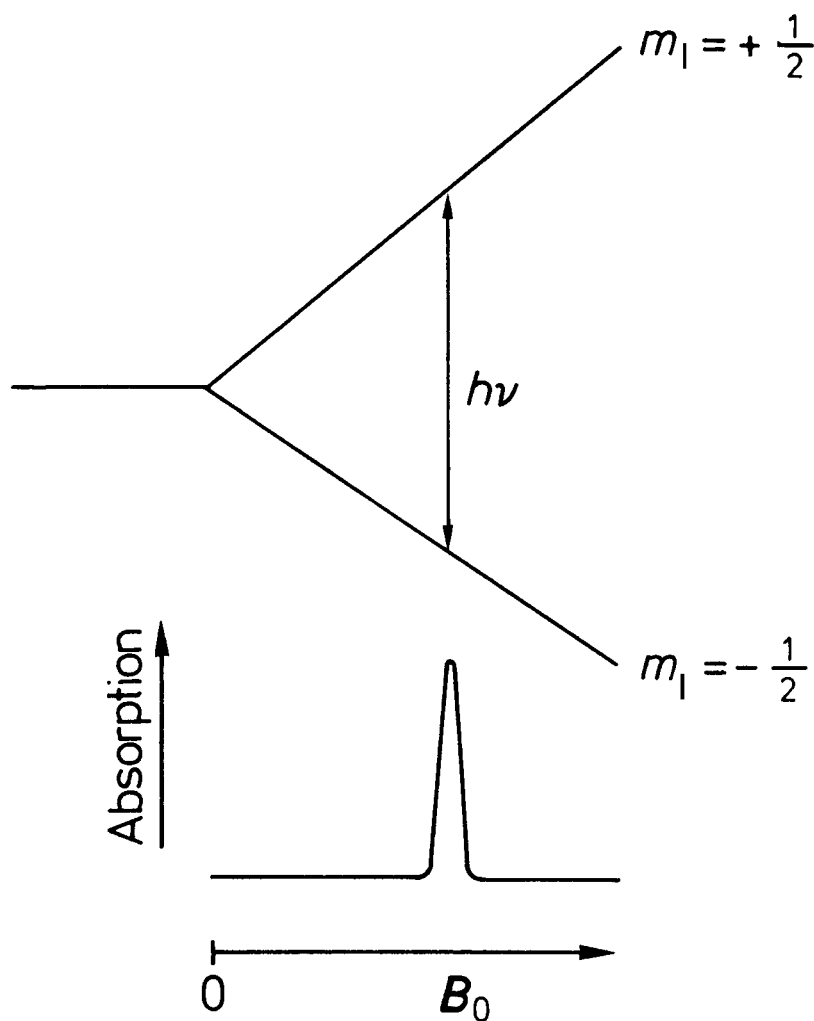
$$\mu_z = +\frac{1}{2}g_N \beta_N \text{ or } \mu_z = -\frac{1}{2}g_N \beta_N. \quad \text{Eq. 6.12}$$

In an external magnetic field, these two orientations of the magnetic dipole moment correspond to two energy levels that are

$$E = -\vec{\mu} \cdot \vec{B} = -\frac{1}{2}g_N \beta_N B_0 \text{ and } E = -\vec{\mu} \cdot \vec{B} = +\frac{1}{2}g_N \beta_N B_0 \quad \text{Eq. 6.13}$$

For a transition from the energetically preferred state to the higher energy level, the sample has to be irradiated by radiation of such energy. The resonance condition is then:

$$\Delta E = g_N \beta_N B_0 = h\nu \quad \text{Eq. 6.14}$$

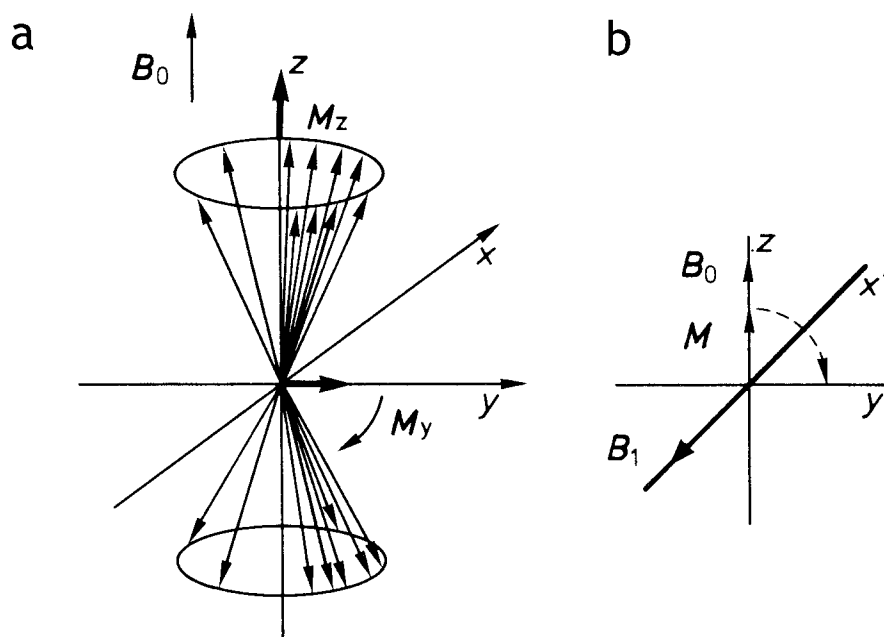


The resonance frequency in a magnetic resonance experiment depends on the strength of the magnetic field B_0 .

Figure 6.3 The resonance frequency depends on the strength of the magnetic field B_0 .

6.1.2 The resonance experiment

The interaction between the spinning nucleus and the incident radiation therefore strictly requires an external magnetic field and the absorption frequency of magnetic resonance is dependent on the strength of the applied magnetic field (illustrated in Figure 6.3). How does resonance take place ?



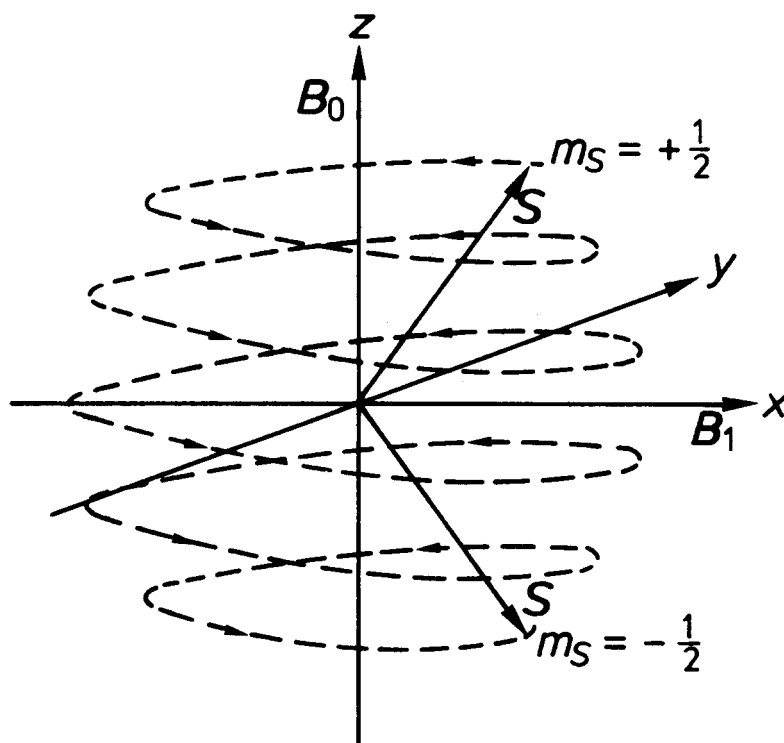
a Effect of a B_1 -field that is oriented in x-direction. The forced phase coherency of the spins induces a transverse magnetization M_y in y-direction.

b Precession of the magnetization along the direction of the B_1 -field (that defines the x' direction of the rotated coordinate system.)

Figure 6.4 Effect of the radio wave B_1 field on the nuclei precessing around the direction of the external B_0 field.

When the external field B_0 is applied, the nuclear spins orient themselves such that they are precessing around an axis that is defined by the magnetic field vector $\vec{B}_0$ with the Larmor frequency. Most nuclei will orient themselves such that their magnetic moment is directed parallel to the $\vec{B}_0$ field vector. Some of them will orient themselves antiparallel to the $\vec{B}_0$ field. The orientation of the nuclear spin in the $\vec{B}_0$ field also defines the direction of the precession of the spin. There is now a macroscopic magnetization M_z in z-direction that is caused by the nuclei of the substance in the field. While the spins are all precessing with the Larmor frequency, there is no phase coherence between them, i.e. their position on the precession cone is random (Figure 6.4). This also means, that there is no magnetization in x or in y direction. In an NMR experiment a linearly polarized radio wave radiation (typically of a frequency between 60 MHz and 1 GHz) is now directed towards the sample. The

magnetic field vector $\vec{B}_1$ of this radiation oscillates in x-direction. In our discussion of CD-spectroscopy, we have seen that a linearly polarized changing magnetic field can be decomposed into two circularly polarized components. These are characterized by two rotating magnetic field vectors in the x-y plane, which rotate in opposite directions. If the resonance condition is met the circularly polarized component that rotates in the direction of the precessing spins will force some of the spins to be phase coherent. The field $\vec{B}_1$ rotates with the Larmor frequency



Superposition of the precessions around the direction of B_0 -field (z-axis) and the B_1 -field. The B_1 -field rotates with the Larmor frequency around the z-axis. Precession around the axis of the B_1 -field is slower because of B_1 is smaller than B_0 .

Figure 6.5 Superposition of the spin precession around the z- and around the x-axes. induced by the magnetic field vectors B_0 and B_1 .

and induces such partial phase coherence and thus a magnetization M_y perpendicular to $\vec{B}_0$ which oscillates in the x, y plane with the Larmor frequency (Figure 6.4). A receiver coil that is positioned along the y-direction detects the voltage that is induced by the rotating magnetization vector (M_y) and detects when absorption of the radiation occurs. To imagine the absorption process we view the coordinate system as rotating with the Larmor frequency around the z-axis. The rotating axes are now designated x' and y' . The advantage of a rotating coordinate system is that now the magnetic field vector $\vec{B}_1$ is stationary in the

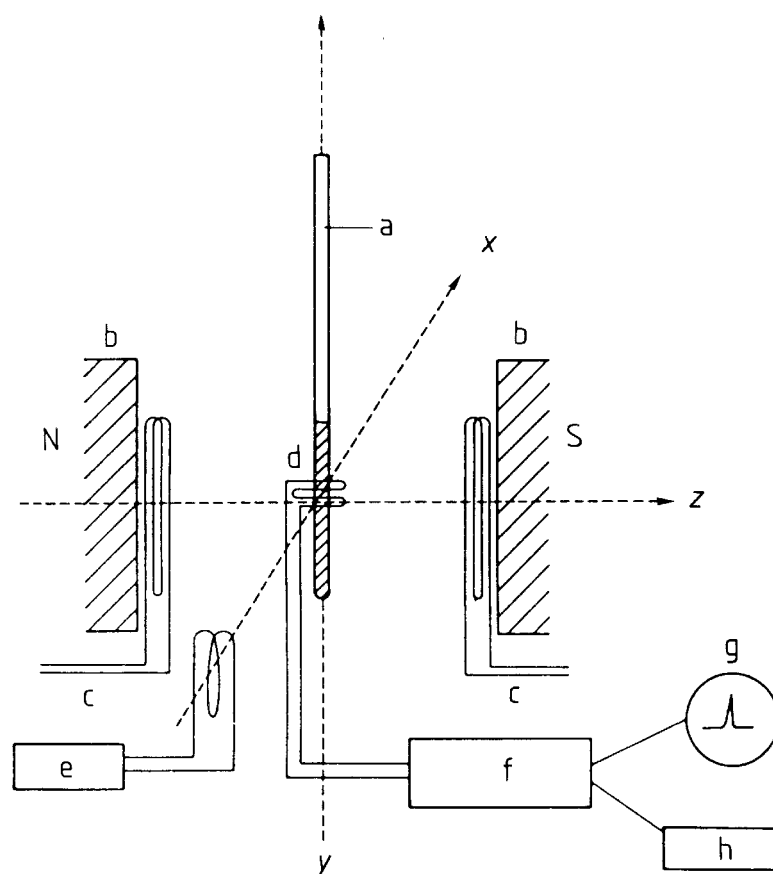
direction of x' . The magnetization in z -direction is affected by the $\vec{B}_1$ field and a torque

$$T = \vec{M}_z \times \vec{B}_1 \quad \text{Eq. 6.15}$$

is exercised, which is perpendicular to the directions of both $\vec{B}_0$ and $\vec{B}_1$ and therefore in y -direction, giving rise to a magnetization in y' -direction (Figure 6.4). As a consequence, more spins are driven to an antiparallel orientation with respect to $\vec{B}_0$. If a resonant $\vec{B}_1$ field is irradiating the sample, a precession of a magnetization vector around the direction of the $\vec{B}_1$ -field axis is induced (Figure 6.5).

6.1.3 The NMR spectrometer.

In practice, the NMR-spectrometer operates at a fixed frequency of the radio wave radiation (i.e. a fixed frequency of the $\vec{B}_1$ field, for instance 500 MHz) and the strength of the B_0 field is altered (field sweep). A scheme of the NMR spectrometer is depicted in Figure 6.6.



Scheme of a continuous wave NMR-spectrometer: a sample, b magnet, c sweep coils, d receiver, e sender, f amplifier, g oscilloscope, h digitizer and computer. In principle, a Fourier transform NMR spectrometer operates similarly, but the sender e is substituted by an impuls generator and f by an array of complex electronic devices.

Figure 6.6 Scheme of a nuclear magnetic resonance spectrometer.

Figure 6.6 shows a scheme of a typical continuous wave NMR spectrometer. The instruments are often named after their operating frequency (e.g. a 500 MHz NMR spectrometer). Modern NMR spectrometers use Fourier transform techniques instead of the older continuous wave operation.

6.2. Structure of an NMR-spectrum

Each NMR spectrum is characterized primarily by two properties. The chemical shift determines how much the absorption line is shifted due to magnetic shielding by the chemical environment of the absorbing nucleus. The chemical shift therefore characterizes the position of the absorption peak relative to an unshielded nucleus. The second property that determines the appearance of an absorption line is the spin-spin coupling, which leads to a multiplet structure of the NMR spectrum. The spin-spin coupling is caused by a magnetic interaction between nuclei in close proximity to each other. The chemical shift and the spin-spin coupling will be observed independent of the nucleus, i.e. these properties are found for ^1H , ^{13}C , ^{15}N , etc. The differences between the nuclei are in the different positions of the resonance lines. For instance if the B_0 -field is 10 T, the ^1H resonance appears at 425.7 MHz, and the ^{13}C resonance at 107.1 MHz. With one experiment (B_0 -field), only one kind of nucleus can be investigated spectroscopically.

6.2.1 Chemical shift

Until now, we have described the NMR experiment for isolated nuclei. However, this is not very realistic, since all nuclei are associated at least with electrons in an atom or molecule. The B_0 -field induces ring currents in the electron cloud in an atom. These ring currents are in turn inducing a magnetic field B_i which is directed against to the B_0 -field. The investigated nucleus is shielded and a stronger field B_0 has to be used for nuclear magnetic resonance to take place. The magnitude of the induced field is dependent on the magnitude of the B_0 -field. The local field is therefore given by

$$B_{loc} = B_0 - \sigma \cdot B_0 = B_0(1 - \sigma) \quad \text{Eq. 6.16}$$

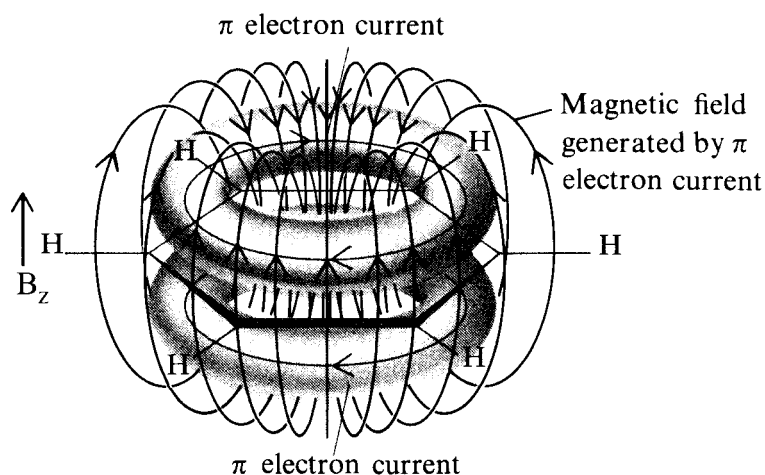
The shielding constant σ is strongly dependent on the electron density, i.e. also on the neighbor atoms of the nucleus. For example, the electron density close to the proton of a methyl group (CH_3) is much larger than the electron density of a proton in a O-H group, because of the higher electronegativity of the oxygen in OH. Therefore the shielding constant will be larger for the proton in OH than for that in CH_3 . Shielding can also be influenced by neighbor groups like ($-\text{CH}_3$, $-\text{NH}_2$, $-\text{COOH}$, etc).

A special shielding effect is observed in benzene (Figure 6.7). The B_0 -field induces a ring-current in benzene. In the center of the ring, the induced magnetic field of the molecule is directed against the B_0 -field, but outside the ring, where the aromatic protons are located, the induced magnetic field has the same direction as the B_0 -field. Thus the field at the protons is enhanced and shielding is weakened. The resonance of such protons occurs at relatively small field. The chemical shift is a characteristic quantity that depends on the chemical environment of the nucleus. The importance of NMR as a tool to probe the chemical environment is thus obvious.

For practical reasons, the chemical shift is expressed independently of the magnetic field B_0 and radiation frequency, by a reference signal:

$$\delta = \frac{\nu_{ref} - \nu}{\nu_{ref}} \cdot 10^6 \text{ ppm} \quad \text{Eq. 6.17}$$

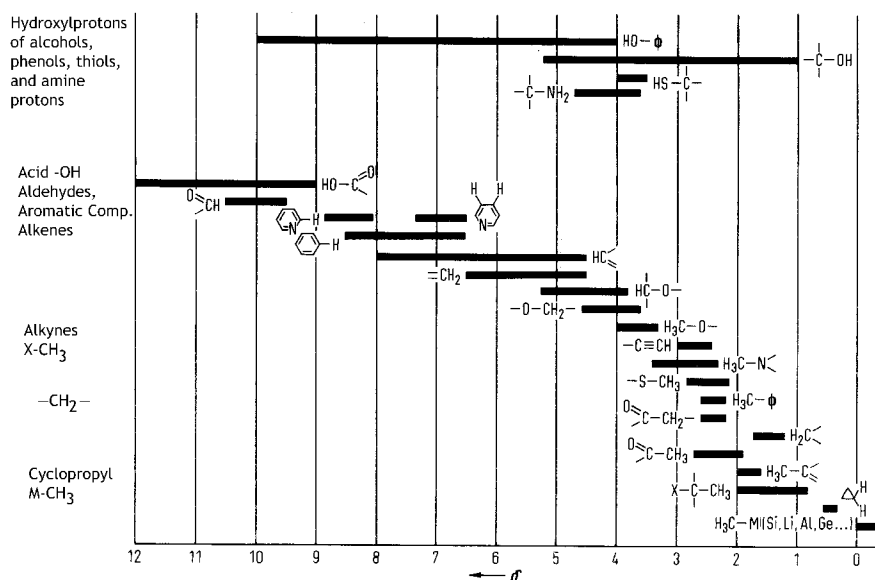
Thus a chemical shift of 300 Hz in a 300 MHz spectrum would have a δ of 1 ppm and a shift of 600 Hz in a 600 MHz spectrum would also be 1 ppm. In ^1H -NMR spectroscopy, the reference signal is that of tetramethylsilane (TMS, $\text{Si}(\text{CH}_3)_4$) in organic solvent or 2,2,-dimethyl-2-silapentane-5-sulfonate (DSS) in aqueous solvent. In comparison to most other protons, the protons in these compounds have a better chemical shielding.



The ring-current effect on the local magnetic field felt by aromatic protons. The applied external field is B_z .

Figure 6.7 Shielding effect by the π -electrons in benzene.

The chemical shift is given in ppm units from the right to the left in a spectrum. Positive numbers characterize a shift of the resonance line to a lower magnetic field B_0 . Some chemical shift values in ^1H -NMR are given in Figure 6.8.

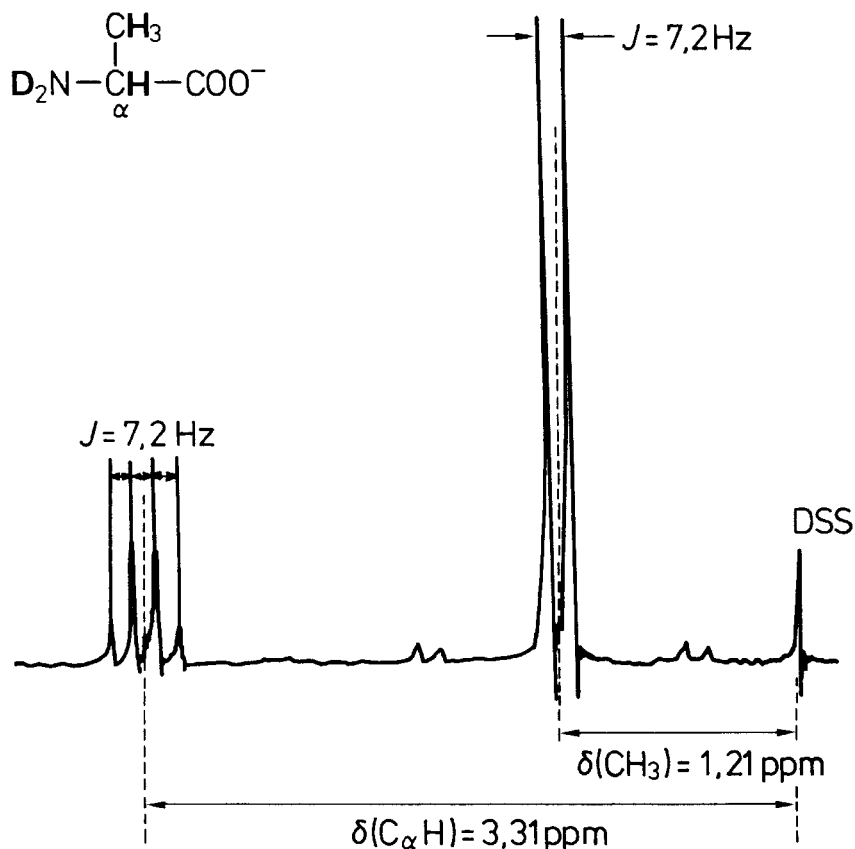
Chemical shifts of various ^1H nuclei of organic compoundsFigure 6.8 Some chemical shift values of proton resonances in ^1H -NMR spectra.

6.2.2 Spin-spin coupling.

In Figure 6.9 the proton NMR spectrum of alanine in D_2O is shown at $\text{pD}=13$. At a pD of 13 all amine protons are exchange by deuterium, D. Because of the chemical shift, the doublet at $\delta=1.21$ ppm can be assigned to the methyl protons and the quadruplet at $\delta=3.31$ ppm can be assigned to the α -carbon hydrogen. The appearance of multiple resonance lines is a consequence of the spin-spin coupling between protons in direct neighborhood, because the carbon atoms are mostly present as ^{12}C , which does not have a spin. We observe a vicinal proton-proton coupling. The magnitude of the proton-proton coupling is expressed by the coupling constant J , which is in the region of 0 to 30 Hz for protons in 100 MHz spectra (approx. 0.2 ppm).

How can the coupling be explained? The 3 protons of the methyl group are magnetically equivalent. Without a spin-spin interaction we would observe one resonance line at 1.21 ppm, because the coupling of equivalent nuclei does not affect the NMR spectrum. The hydrogen at the neighbor α -carbon can be in two different spin states with $m_1=-1/2$ or $m_1=+1/2$. The corresponding magnetic moments of these spins lead to a local decrease or increase of the magnetic B_0 field. The NMR signal of the methyl protons is split into a doublet. An equivalent

consideration leads to the quadruplet of the NMR resonance line of the hydrogen at the α -carbon atom. The spins of the three individual protons can be combined to a total spin that can be either $+3/2$, $+1/2$, $-1/2$, and $-3/2$. We obtain two different field amplifications and two different field attenuations, therefore, 4 resonance lines. From this and similar considerations



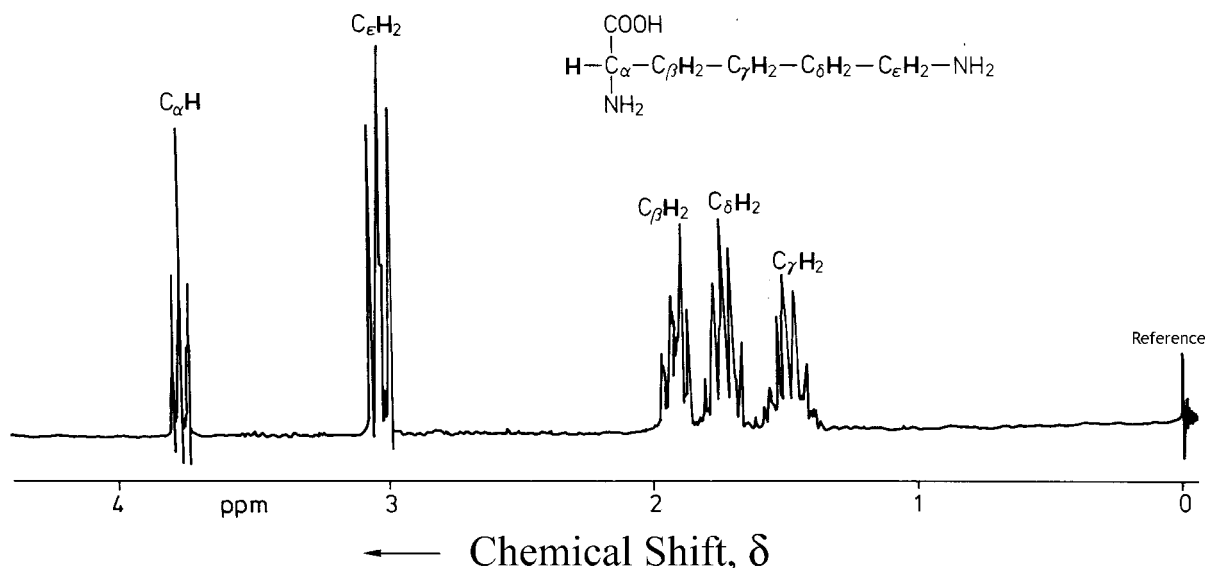
^1H -NMR spectrum of alanine

Figure 6.9 Proton NMR spectrum of alanine

we can derive the general rule that n equivalent nuclei with spin $1/2$ in direct neighborhood of a proton lead to $n+1$ multiplet components in the NMR spectrum of this proton. The intensities of these peaks can be derived from Pascals triangle:

$n=0$					1					
$n=1$				1		1				
$n=2$			1		2		1			
$n=3$		1		3		3		1		
$n=4$	1		4		6		4		1	

In case of the C- α hydrogen, the observed intensities of individual bands in the multiplet are 1:3:3:1. Multiplets with superpositions arise from couplings with more than one set of nuclei. In Figure 6.10 this is shown on the example of the amino acid lysine.



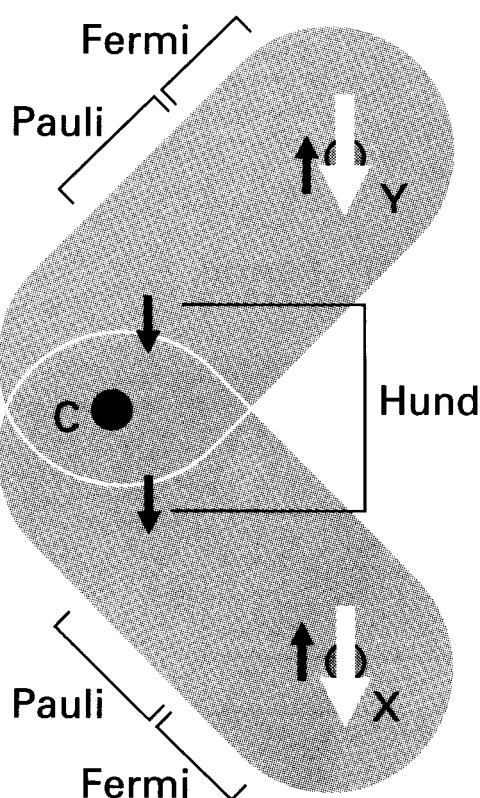
^1H -NMR spectrum of lysine at 220 MHz

Figure 6.10 Proton NMR spectrum of lysine at 220 MHz.

The scalar coupling that has been observed in the two examples of alanine and lysine is also called J-coupling or indirect coupling, because it is mediated by the bonds between the nuclei. Direct coupling or through-space coupling is also observed, but mostly in the solid phase. In liquids, the direct coupling is often averaged to zero because of the fast rotational motion of the molecules.

The scalar coupling constant is given by the splitting of a resonance line and given in the frequency unit Hertz (Hz). Different type of scalar coupling are distinguished. The scalar coupling constant of two nuclei that are separated by N bonds is denoted $^N J$, with subscripts of the type of nuclei involved. Thus, $^1 J_{\text{CH}}$ is the coupling of a proton directly joined to a ^{13}C atom. $^2 J_{\text{CH}}$ is the coupling constant when the same nuclei are separated by 2 bonds, such as in ^{13}C -C-H. A typical value of $^1 J_{\text{CH}}$ is in the range of 120 to 250 Hz. The coupling constant is a consequence of the nuclear magnetic moment and therefore independent of the external B0 field. Therefore the coupling constant is given in Hz. $^2 J_{\text{CH}}$ is between 0 and 10 Hz. Both 3J and 4J give detectable effects in a spectrum, but couplings over larger numbers of bonds can generally be ignored. The sign of the coupling indicates whether the energy of the two spins is lower when they are parallel or when they are antiparallel. Spin-spin coupling in molecules in solution can be explained in terms of the polarization mechanism, in which the interaction

is transmitted through the bonds. The simplest case is to consider $^1J_{XY}$ where X and Y are spin 1/2 nuclei joined by an electron pair bond. The coupling mechanism depends on the fact that in some atoms it is favorable for the nucleus and a nearby electron spin to be both parallel (both α or both β), but in others it is favorable for them to be antiparallel (one α , one β). The electron-nucleus interaction is magnetic in origin, and may be either a dipolar interaction between the magnetic moments of the electron and nuclear spins or a Fermi contact interaction. The Fermi contact interaction depends on the very close approach of the electron to the nucleus and can occur only if the electron occupies an s orbital. The Fermi



The polarization mechanism for $^2J_{HH}$ spin-spin coupling. The spin information is transmitted from one bond to the next by a version of the mechanism that accounts for the lower energy of electrons with parallel spins in different atomic orbitals (Hund's rule of maximum multiplicity). In this case, J is negative, corresponding to a lower energy when the nuclear spins are parallel.

Figure 6.11 Indirect spin-spin coupling mechanism between geminal hydrogens.

contact interaction is a consequence of the different magnetic field pattern that is present close to the nucleus. It is usually energetically favorable for a nucleus and an electron to be antiparallel. If the X nucleus is an α , a β electron of the bonding pair will tend to be found nearby (since that is energetically favorable for it). The second electron in the bond, which must have an α -spin if the other is β , will be found mainly at the far end of the bond because electrons tend to stay apart from each other. Because it is energetically favorable for the spin of Y to be antiparallel to an electron spin, a Y nucleus with a β -spin has a lower energy and hence a lower Larmor frequency than a Y nucleus with an α spin. The opposite is true for an X with β , for now the α -spin of Y has the lower energy. In other words, the antiparallel arrangement of nuclear spins lies lower in energy as the parallel arrangement as a result of their magnetic coupling with the bond electrons. That is $^1J_{\text{HH}}$ is positive. To account for the value of $^2J_{\text{XY}}$, as in HCH, we need a mechanism that can transmit the spin alignments through the central C-atom, which may be ^{12}C with no nuclear spin of its own. An X-nucleus with an α -spin polarizes the electrons in its bond and the α -electron will be found closer to the C-nucleus. The more favorable arrangement of two electrons on the same atom is with their spins parallel (Hunds rule). So the more favorable arrangement is that of the electron with the α -spin to be near the C-nucleus. Consequently, the β -electron will be closer to the Y-nucleus, which in turn will have a lower energy if it has an α spin, i.e. its spin will be parallel to that of the X-nucleus and the coupling constant will be negative.

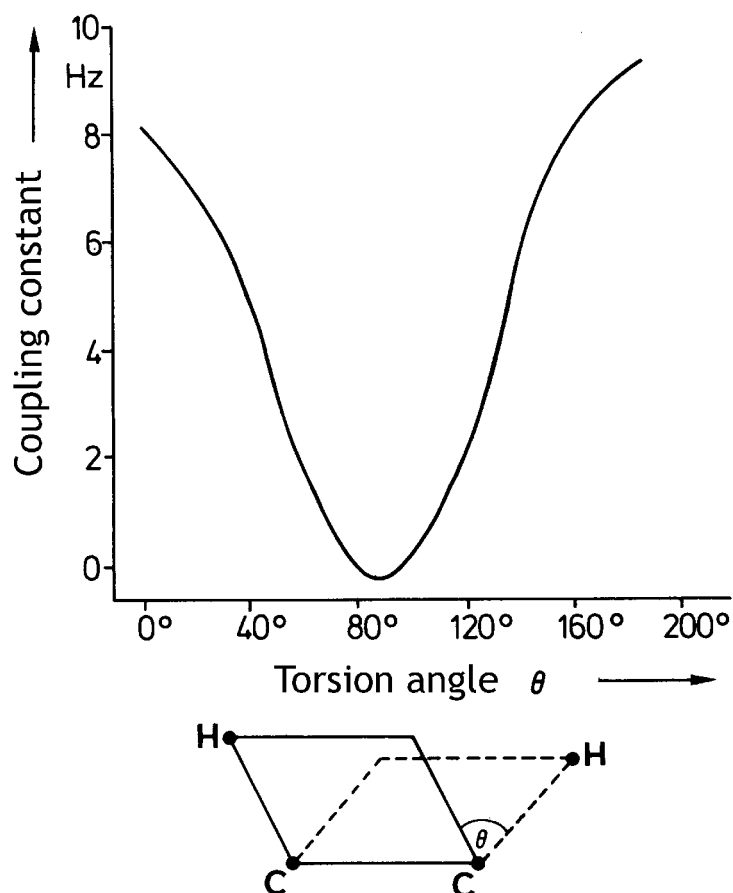
The coupling of a nuclear spin to an electron spin by the Fermi contact interaction is most important for proton spins, but is not necessarily the most important mechanism for other nuclei. These nuclei may also interact by a dipolar mechanism with the electron magnetic moments and with their orbital motion, and there is no simple way of specifying whether J will be positive or negative.

The vicinal H,H coupling, $^3J(\text{H,H})$, has been well investigated, since it is found in saturated hydrocarbons. It was found that the coupling is dependent on several factors:

- the torsion angle
- the substituents
- the C-C distance
- the H-C-C- valence angle

As an example the dependence of the $^3J(\text{H,H})$ coupling on the torsion angle is depicted in Figure 6.12. The coupling as a function of the torsion angle is described by the Karplus equation:

$$J = A + B \cos(\phi) + C \cos(2\phi) \quad \text{Eq. 6.18}$$



Dependence of the coupling constant on the torsion angle between two hydrogen atoms.

Figure 6.12 Dependence of the vicinal $3J(\text{H},\text{H})$ coupling on the torsion angle.

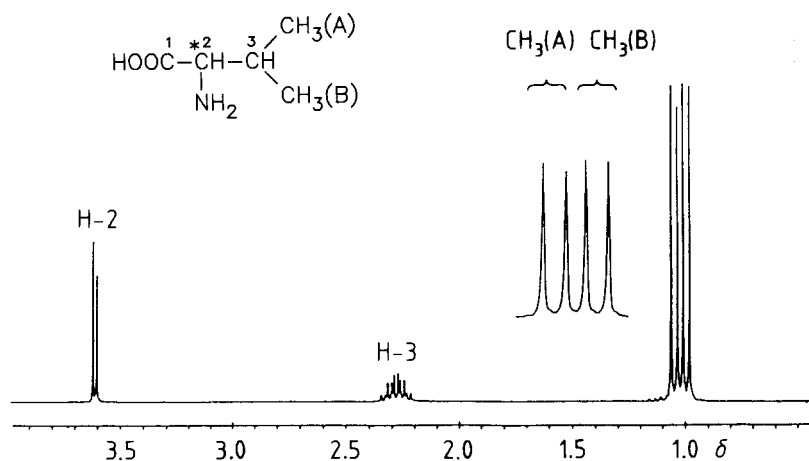
in which $A \approx +7 \text{ Hz}$, $B \approx -1\text{Hz}$, $C \approx +5\text{Hz}$, respectively.

It has already been mentioned that magnetically equivalent protons do not couple among each other. To describe the coupling between magnetically distinct nuclei a nomenclature was introduced in which nuclei with very distinct chemical shifts are described by letters A and X. Nuclei with chemical shifts close to each other are described by letters A and B. We shall first consider an AX spin system, in which both nuclei have the spin $1/2$. Suppose the spin of X is α ; then the spin of A will have a Larmor frequency as a combined effect of the external field, the shielding constant and the spin-spin interaction with X. The spin-spin coupling will

result in one line in the spectrum of A that is being shifted from A by $-1/2 J$ from the frequency it would have in the absence of coupling. If the spin of X is β , the spin of A will have a Larmor frequency shifted by $+1/2 J$. Therefore, instead of a single line from A, we get a doublet of lines separated by J and centered on the chemical shift characteristic of A. The same splitting occurs in the X-resonance. Instead of a single line, the resonance is a doublet with splitting J (the same value as in the splitting of A) centered on the chemical shift characteristic of X. A subtle point is that the X-resonance in an AX_n spin-system (such as an AX_2 or an AX_3 species) is also a doublet with splitting J . A group of equivalent nuclei resonates like a single nucleus. The only difference is that the peak intensity is n times higher in an AX_n spin system than in an AX spin system. The A resonance in an AX_n system is quite different from the X resonance in an AX system. In an AX_2 spin system, for example three resonance lines are observed because the two X spins can be either all α , $\alpha\beta$, or all β .

An example for an AX_3 spin system was the spectrum of alanine in $D_2O/NaOD$ (Figure 6.9), in which the α -carbon hydrogen resonance is a quadruplet and the resonance of the methyl protons is split into a doublet.

In order to classify a spin system, it is first important to determine the chemically different nuclei and for that purpose the stereo chemistry of the nuclei must be analyzed. In 1H -NMR spectroscopy, homotopic protons are chemically equivalent and give rise to one signal in an isolated group. Enantiotopic protons, for example those in bromochloromethane, are stereochemically not equivalent, but magnetically equivalent, they cannot be distinguished in an NMR spectrum. However, the protons of diastereotopic compounds, for example those in the two methyl groups of valine (Figure 6.13), have a different chemical environment (there is no symmetry operation or bond rotation that could convert these protons into each other).

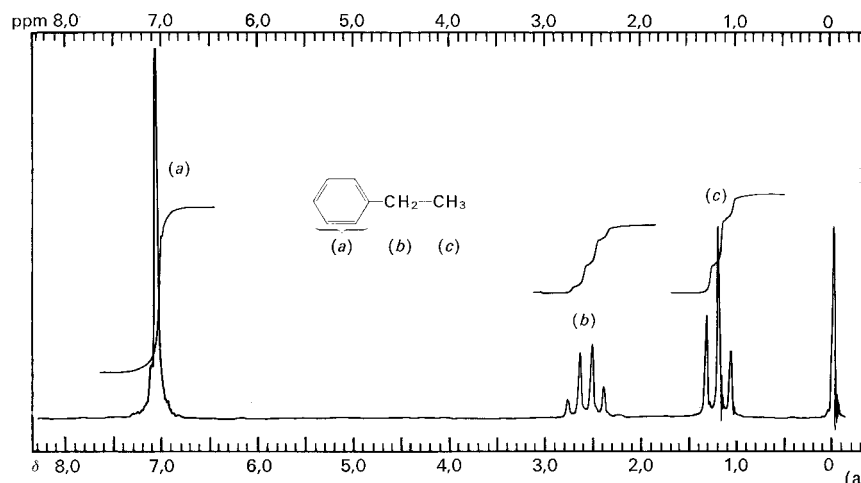


1H -NMR spectrum of valine. The C_α carbon atom is asymmetric. Thus, the two methyl groups on C_β are diastereotopic and give different resonance signals at different chemical shifts. Two doublets are observed because of their different coupling with the C_β -proton.

Figure 6.13 1H -NMR spectrum of valine.

6.3. Examples of ^1H NMR spectra of aromatic compounds.

As discussed before, aromatic hydrogen atoms show characteristic chemical shift of about $\delta = 6.5$ to 8.5 ppm. Figure 6.14 shows the spectrum of phenylethane. The aromatic protons exhibit a chemical shift of 7.2 ppm while the methylene protons appear at $\delta \approx 2.6$ ppm and the methyl protons appear at $\delta \approx 1.2$ ppm. The methyl protons appear as a triplet and the methylene protons appear as a quadruplet due to spin-spin coupling.



^1H -NMR spectrum of ethylbenzene. The triplet of the methyl protons (c), the quadruplet of the methylene protons (b) and the peak of the aromatic protons (a) are observed in the direction of increased chemical shift.

Figure 6.14 ^1H -NMR spectrum of phenylethane

The spectrum of Figure 6.14 was obtained at relatively low frequency and is not very well resolved. Thus the differences among the aromatic protons are not very well resolved and only a single peak appears in the spectrum. On a better NMR spectrometer, differences between aromatic protons can be observed and we shall discuss two examples.

In aniline the aromatic protons H2 and H6, H3 and H5 and H6 are different as is evident from the electron densities in the aromatic ring (Figure 6.15). Because of the +M effect of the amino group the electron density is higher at carbon atoms 2,4, and 6 than at carbon atoms 3 and 5. The magnetic shielding of H4 is therefore different from that of H2 and H6. The resonances of H3 and H5 have the lowest chemical shift. The splitting of the resonance lines is complex. The resonance lines of the magnetically equivalent H2 and H6 are split by a J-coupling with the other aromatic protons, and largely appear as a doublet, since the coupling between H2 and H3 (or H5 and H4) is strongest. The resonance line of H4 is split most effectively by the magnetically equivalent protons H3 and H5 into a triplet although there is fine structure by J-coupling over more than 3 bonds. The coarse structure of the resonances

of H3 and H5 may also be described as a triplet. However the protons H2 and H4 that strongly couple with H3 are not magnetically equivalent. Overall the splittings of the aromatic resonances are far more difficult to interpret than those of relatively isolated methyl or methylene groups in phenylethane.

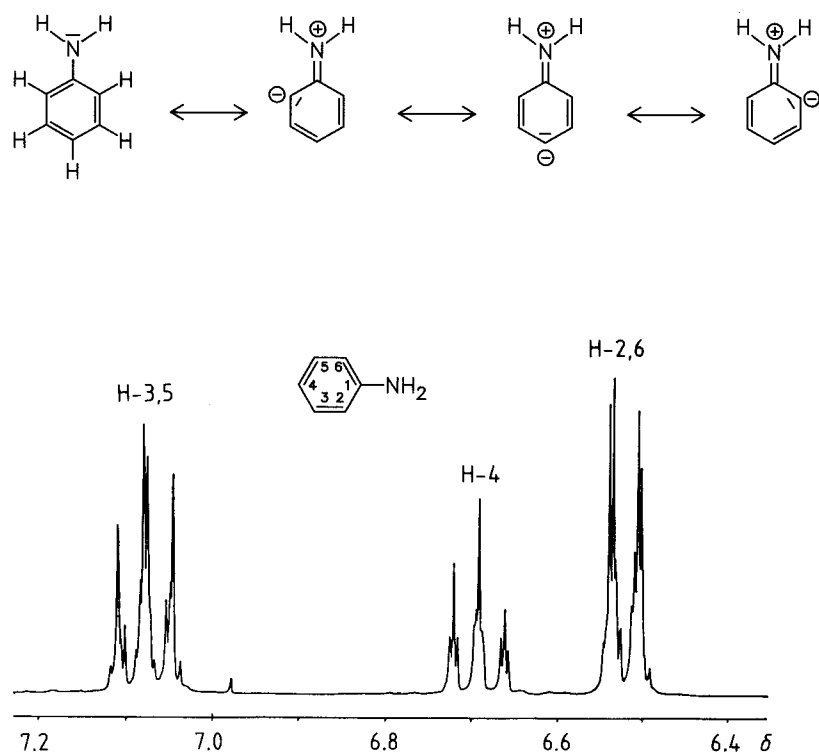


Figure 6.15 250 MHz ¹H-NMR spectrum of aniline in CDCl₃ (δ(NH₂) = 3.45 ppm)

Another example of the chemical shift of aromatic protons is given in Figure 6.16. In nitrobenzene, the nitro group has a -M effect. Thus, the electron density is lower at hydrogens 2, 4 and 6 than it is at hydrogens 3 and 5. Hydrogens 3 and 5 are better shielded and their resonances appear at lower chemical shifts than those of H2, H4, and H6.

Overall the chemical shifts are larger than in the previous sample of aniline, because of the stronger deshielding of the aromatic protons by the nitrogen substituent.

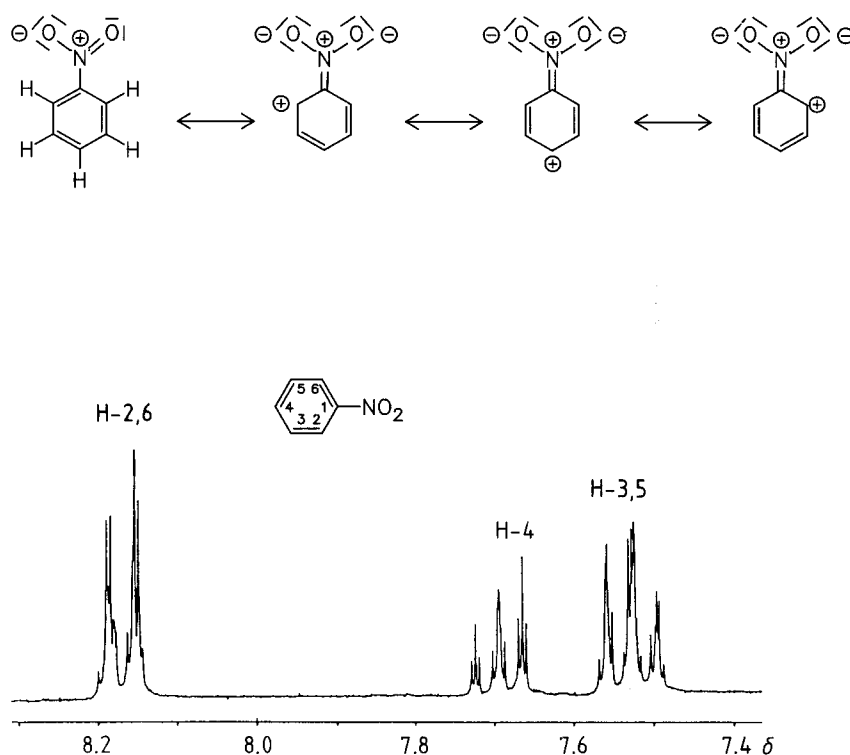


Figure 6.16 250 MHz ¹H-NMR spectrum of nitrobenzene in CDCl₃.

Numerous experiments with benzene derivatives have shown that the substituents contribute nearly invariably constant changes to the chemical shifts of aromatic protons. These contributions to the chemical shift have been determined from experimental data. With their help and the chemical shift value for the protons of benzene (7.27) it is possible to assign the chemical shifts of many aromatic protons of benzene derivatives.

$$\delta(H) = 7.27 + \sum S \quad \text{Eq. 6.19}$$

The substituents increments are given for some substituents in the Table 2 below:

Example: p-nitroanisole

$$\begin{aligned} \delta(H-2, 6) &= 7.27 + S_o(\text{OCH}_3) + S_m(\text{NO}_2) \\ &= 7.27 - 0.43 + 0.17 = 7.01 \text{ (found: 6.88)} \end{aligned}$$

$$\begin{aligned} \delta(H-3, 5) &= 7.27 + S_o(\text{NO}_2) + S_m(\text{O-CH}_3) \\ &= 7.27 + 0.95 - 0.09 = 8.13 \text{ (found: 8.15)} \end{aligned}$$

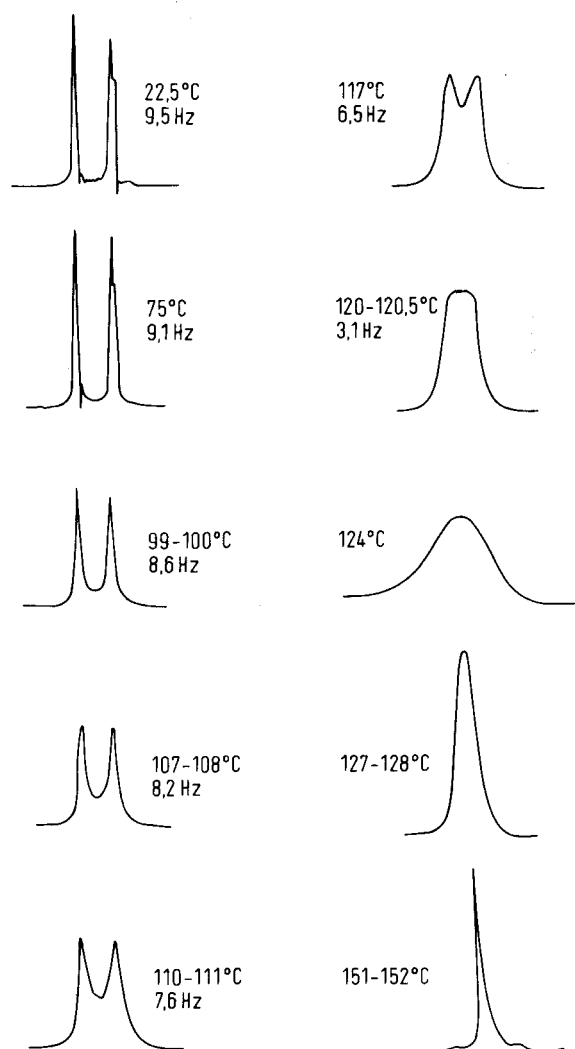
in addition $\delta(\text{OCH}_3) = 3.90$

	ortho	meta	para
Methyl–	–0.17	–0.09	–0.18
Ethyl–	–0.15	–0.06	–0.18
Fluoro–	–0.30	–0.02	–0.18
HO–	–0.50	–0.14	–0.4
–O-CH ₃	–0.43	–0.09	–0.37
–OCOCH ₃	–0.21	–0.02	–0.0
–NH ₂	–0.75	–0.24	–0.63
–N(CH ₃) ₂	–0.60	–0.10	–0.62
–CHO	+0.58	+0.21	+0.27
–COCH ₃	+0.64	+0.09	+0.3
–COOCH ₃	+0.74	+0.07	+0.20
–NO ₂	+0.95	+0.17	+0.33

Table 2 Contributions of different substituents to the chemical shifts of aromatic protons.

6.4. Coalescence of spin-spin coupling

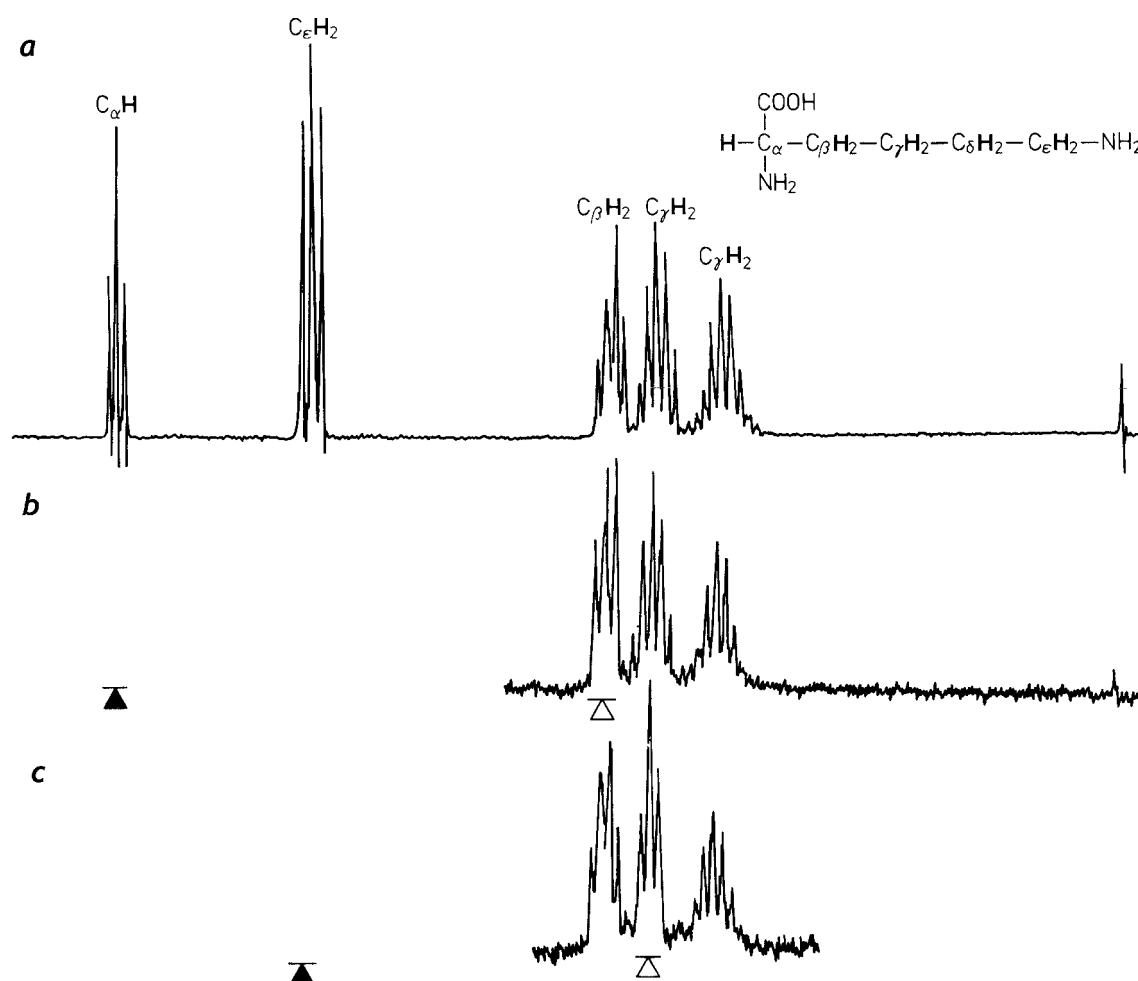
The NMR signals are dependent on the possibility of exchange of nuclei. In dimethyl formamide for example, the NMR spectrum consists of two signals of methyl protons that are observed at $\delta = 2.79$ ppm and at $\delta = 2.94$ ppm at 22.5 °C. These two signals are broadened above +100 °C and coalesce at +120 °C to one broad band (Figure 6.17). Upon further temperature increase, this band becomes sharper and positioned in the center of the original peaks. From the temperature dependence of the ^1H resonances it is found that the methyl groups are differently shielded at room temperature. The difference is caused by a restricted rotational mobility of the C-N bond, that contains a significant contribution of p-electrons from the nitrogen, leading to a partial double bond character of the C-N bond. At higher temperature (energy), rotation around the C-N bond is possible and the methyl groups become equivalent. The temperature at which the exchange between the nuclei of different chemical shifts becomes possible is called the coalescence temperature.



^1H -NMR signals of the methyl groups in dimethyl formamide, recorded at different temperatures (frequency: 56.4 MHz). At slow exchange, two signals are obtained, at fast exchange only one signal. In the intermediate region, the signals are broadened. The coalescence temperature is 120 °C.

Figure 6.17 Coalescence of NMR signals.

6.5. Spin-Spin-decoupling and broad-band (BB) decoupling

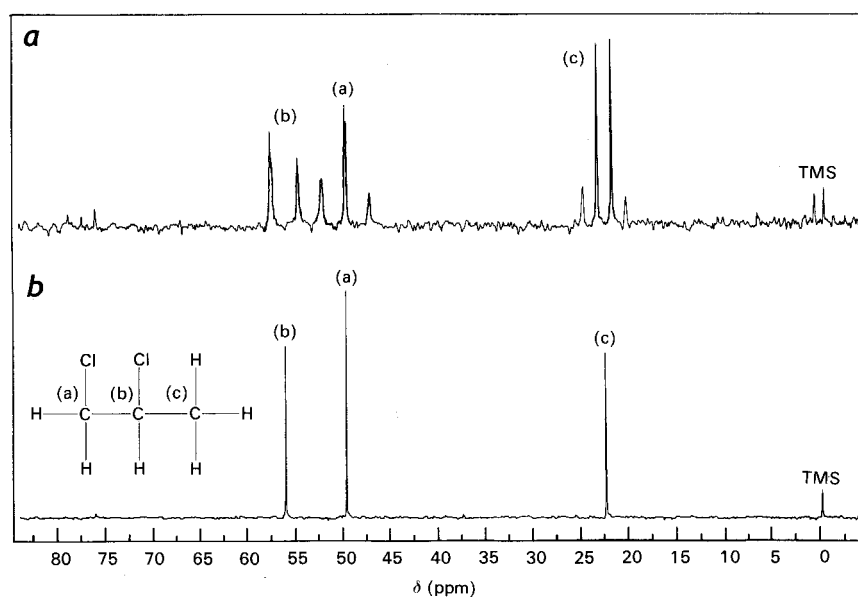


Spin-spin decoupling on the example of lysine in D_2O . **a** not decoupled. **b** decoupling of the C_{α} protons. **c** decoupling of the C_{δ} protons. The frequencies of decoupling are indicated by the black arrows. The white arrows indicate changes in the multiplicities that are observed in when neighbor protons are decoupled.

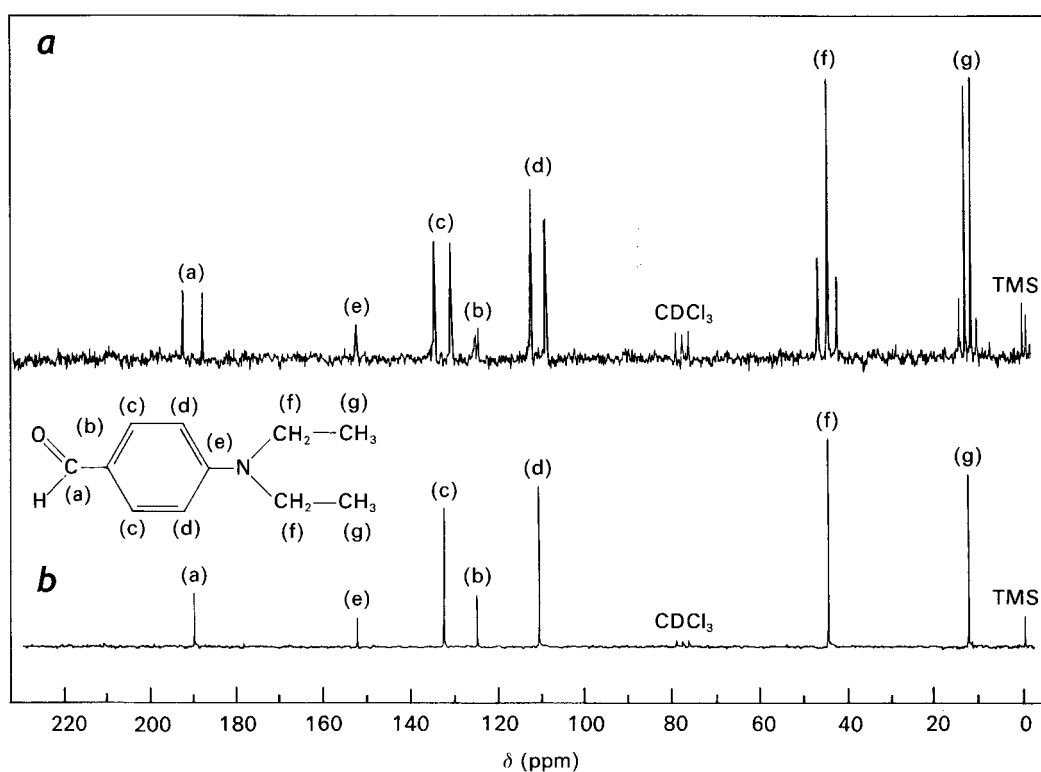
Figure 6.18 Spin-Spin decoupling of the lysine NMR peaks.

The spin-spin coupling between two chemically distinct spins may be eliminated if the transition of one of these spins is saturated by irradiating the sample with the resonance frequency of this spin. Now the ground state and the excited state of this spin are equally populated and *there are continuously transitions between these states*. Now, this nucleus does not longer contribute to the local magnetic field at the neighbor nucleus, such that the signal of the neighbor nucleus is now not split. For this method, a second sender (decoupler) is necessary, which irradiates the nuclei that are to be decoupled. Figure 6.18 displays the spectrum of lysine with decoupling of the C_{α} (b) and the C_{ϵ} protons (c). Changes in the spectrum are marked by arrows. Similarly, in ^{13}C -NMR spectra, all ^1H couplings may be “broad-band decoupled” by irradiating with a “white” frequency spectrum in the region of the

proton resonances.



a ^{13}C -NMR spectrum of 1,2-dichloropropane. **b** Proton-decoupled ^{13}C -NMR spectrum of 1,2-dichloropropane.



a ^{13}C -NMR spectrum of p-diethylaminobenzaldehyd (in CDCl_3).
b Proton decoupled ^{13}C -NMR spectrum of p-diethylaminobenzaldehyd (in CDCl_3).

Figure 6.19 ^{13}C NMR spectra of p-diethylaminobenzaldehyd.

6.6. Resonance frequencies of some nuclei with non-zero spin

Given the resonance condition

$$\Delta E = g_N \beta_N B_0 = h\nu \quad \text{Eq. 6.20}$$

the frequency at which the nuclear magnetic resonance will occur in a given field B_0 , will depend on the properties of the nucleus (or if the electron is considered, on the spin and magnetogyric ratio of the electron). Typical resonance frequencies of various nuclei in a field of $B_0 = 2.3487$ T are shown in Table 3.

Nucleus	Spin	Resonance Frequency (MHz) field of 2.3487 T	g-value
^1H	1/2	100.00	5.585
^{10}B	3	10.75	0.6002
^{11}B	3/2	32.08	1.792
^{13}C	1/2	25.14	1.404
^{14}N	1	7.22	0.4036
^{15}N	1/2	10.13	-0.5660
^{17}O	5/2	13.56	-0.7572
^{19}F	1/2	94.07	5.255
^{29}Si	1/2	19.87	-1.110
^{31}P	1/2	40.48	2.261
^{35}Cl	3/2	9.8	0.5472
e	1/2	65752.8	2.000323

Table 3 Resonance frequencies of various nuclei.

6.7. Relaxation

6.7.1 Boltzmann distribution of spins in a magnetic field.

Transitions between the different energy levels the nuclei have in an external magnetic field B_0 take place in either direction, from the ground state to the excited state or from the excited state to the ground state. For a nucleus with spin $I = 1/2$, this means the z-component of that spin can change from $m_I = +1/2$ to $m_I = -1/2$ or from $m_I = -1/2$ to $m_I = +1/2$. However, the number of absorption and emission processes will depend on the number of spins in the

two energy levels, which nuclei with spin 1/2 can occupy in presence of the external field B_0 . The Boltzmann distribution describes the relation between the number of spins found in the lower and upper energy levels:

$$\frac{n_h}{n_l} = \exp\left(-\frac{\Delta E}{kT}\right) = \exp\left(-\frac{g_N \beta_N B_0}{kT}\right) = \exp\left(-\frac{g_N \hbar \gamma B_0}{kT}\right) \quad \text{Eq. 6.21}$$

The magnetogyric ratio $\gamma = \frac{Ze}{2m}$ is specific for a nucleus and thus the ratio of the number of spins in the excited state to the number of spins in the ground state is dependent on the which species of nuclei is investigated. For example in a field of $B_0 = 10$ Tesla at $T=300$ K:

$$^1\text{H}: Z=1, e=1.6019\text{e-}19 \text{ As}, m=1.67262\text{e-}27 \text{ kg}. \gamma = \frac{Ze}{2m} = 4.7894\text{e}7 \text{ As/kg}, g_N = 5.585$$

$$\frac{n_h}{n_l} = 0.999932$$

$$^{13}\text{C}: Z=6, e=1.6019\text{e-}19 \text{ As}, m=2.1760\text{e-}26 \text{ kg}. \gamma = \frac{Ze}{2m} = 2.20892\text{e}7 \text{ As/kg}, g_N = 1.404$$

$$\frac{n_h}{n_l} = 0.999992$$

$$e: Z=1, e=1.6019\text{e-}19 \text{ As}, m=9.1095\text{e-}31 \text{ kg}. \gamma = \frac{Ze}{2m} = 8.794\text{e}10 \text{ As / kg}, g_e=2.000232$$

$$\frac{n_h}{n_l} = 0.956203$$

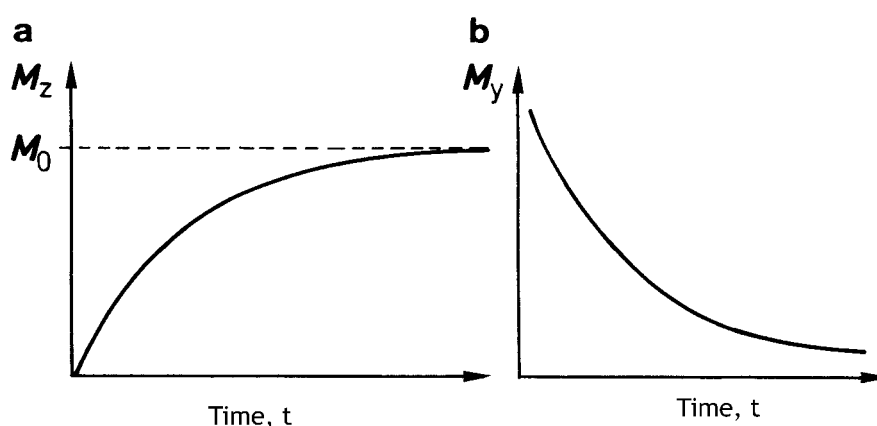
The absorption of radiation is proportional to the difference between the populations of the ground state and the excited state. Thus, the NMR-spectroscopy of the proton is far more sensitive than the NMR spectroscopy of ^{13}C , and NMR methods are far less sensitive than the ESR method.

6.7.2 Relaxation mechanisms.

The difference between the populations of the ground state and the excited state in thermal equilibrium as it is given by the Boltzmann distribution, is changed when radiation is absorbed. This will finally result in that the populations in the excited state and in the ground state will be equally populated and the signal would disappear. This does not happen, because of relaxation mechanisms that lead to the transitions of nuclei in the excited spin state (parallel to the field B_0) to the ground state.

Two mechanisms of relaxation can be distinguished, the spin-lattice relaxation (longitudinal

relaxation) and the spin-spin relaxation (transverse relaxation). We have seen in section 6.1.2 that the transition of the spins that are oriented parallel to the external magnetic field B_0 and therefore result in a macroscopic magnetization in z-direction (field direction), to the excited state takes place by irradiation with polarized radiowaves. Thus a transverse magnetization in the direction of the x-y plane is observed, corresponding to the phase coherence of the precessing spins and an increased number of spins that have antiparallel orientation towards the magnetic field B_0 . If the radio wave (field B_1) is turned off, the longitudinal magnetization M_z in the direction of the external field B_0 will be restored after some time, due to molecular motions. And after some other time the phase coherence of the precessing spins will be lost, due to the magnetic dipole-dipole interaction with neighboring molecules. The first relaxation process, the longitudinal or spin-lattice relaxation, is characterized by the longitudinal relaxation time T_1 , which is the time after which the fraction $1/e$ of the excited spins have returned to the ground state or restored the longitudinal magnetization M_z . The second relaxation process is defined by the transverse relaxation time or spin-spin relaxation time, after which the fraction $1/e$ of the precessing spins has lost its phase coherence (i.e. its transverse magnetization M_{xy}). The spin-spin relaxation affects the lifetime of a spin-state but not the population ratio of the excited and the ground state. The two functions for the return of the magnetization M_z and the decay of the transverse magnetization M_{xy} are shown in Figure 6.20



Time dependence of the change in the magnetization after shutting down the B_1 -field. **a** longitudinal magnetization, M_z . **b** transverse magnetization M_y .

Figure 6.20 Return of the magnetization M_z and decay of the magnetization M_y

and given by the function

$$\frac{dM_z}{dt} = -\frac{1}{T_1}(M_z - M_{z0}), \quad \text{Eq. 6.22}$$

integrated $M_z = M_{z0} \left[1 - \exp\left(-\frac{t}{T_1}\right) \right]$ Eq. 6.23

for spin-lattice relaxation and by the functions

$$\frac{dM_x}{dt} = -\frac{1}{T_2} M_x; \quad \frac{dM_y}{dt} = -\frac{1}{T_2} M_y;$$

integrated $M_{xy} = M_{xy0} \exp\left(-\frac{t}{T_2}\right)$ Eq. 6.24

For a given nucleus, the spin-lattice relaxation and the spin-spin relaxation may be either equal in magnitude or quite different. In small molecules in solution or in the fluid phase it is often $T_1 \sim T_2$. In the solid state or in macromolecules in solution it is often $T_1 > T_2$ (the transverse magnetization declines before the equilibrium Boltzmann distribution is reached).

In CW NMR spectroscopy, the effect of B_1 on the magnetization is always compensated by relaxation processes.

6.8. Pulse Fourier Transform NMR

In CW-NMR spectra were recorded upon continuous irradiation of the sample with a field B_1 at a constant frequency and by variation of the external magnetic field B_0 . The interpretation of the spectra was characterized by the chemical shift and the spin-spin-coupling constants J . The NMR spectroscopy was significantly extended by the introduction of the pulse Fourier transform spectroscopy (FT spectroscopy). In pulse Fourier transform spectroscopy, additional information is obtained by determining the two relaxation times.

In the FT-NMR experiment, the B_1 field is generated as a pulse, i.e. it is only switched on for a short moment and the behavior of the spin-system is measured in the time domain. The Fourier transformation is then applied to the time domain spectrum to generate the frequency domain spectrum. The procedure was already described in context with IR spectroscopy and the same principles apply in NMR spectroscopy. A condition is that the short high frequency pulse contains all frequencies from the range of chemical shifts that the investigated nucleus may have. We now have an NMR instrument that will give us spectrum within one second.

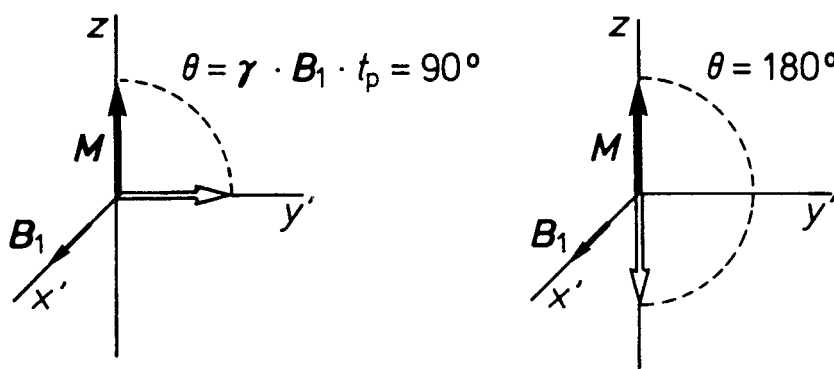
By accumulation of spectra with a computer, a better spectrum can be obtained with Fourier transform NMR spectroscopy than with CW- NMR spectroscopy. The gain in intensity is more than a factor 100 for a given accumulation time compared to continuous wave spectroscopy. The Fourier transformation and analysis of the spectra is performed after accumulation of many scans in the time domain.

6.8.1 Pulse technique.

We have described the impact of the field B_1 in Figure 6.4 as a precession of the longitudinal magnetization around the x' -axis of a rotating coordinate system (x', y', z'). The basis of the pulse technique is that a high frequency pulse in the direction of the x' axis with a duration t_p and with a magnetic induction B_1 will cause a rotation of the magnetization M_z in the zy -plane by a defined angle Θ :

$$\Theta = \gamma \cdot B_1 \cdot t_p \quad \text{Eq. 6.25}$$

The magnetic field B_1 must be chosen such that t_p is small compared to the relaxation times. With typical values of t_p between 1 and 50 μs , it is possible to obtain any angle Θ .



90° and 180° degree pulse.

Figure 6.21 90° and 180 °pulse.

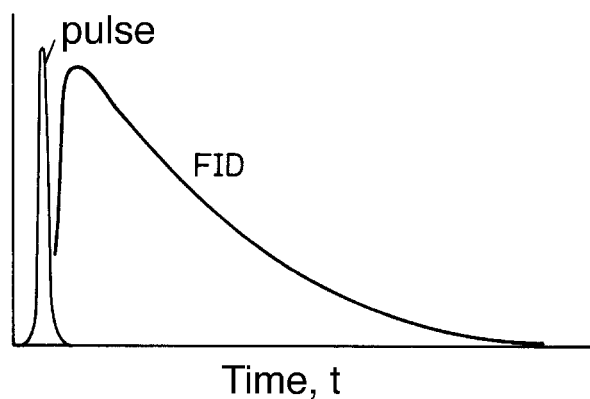
The proportionality constant γ is the magnetogyric ratio that is characteristic for the investigated nucleus. Is the angle Θ by which the magnetization is turned equal 90 ° the pulse is called a 90° pulse, if it is 180 ° the pulse is called a 180 ° pulse (Figure 6.21).

6.8.2 Free induction decay (FID).

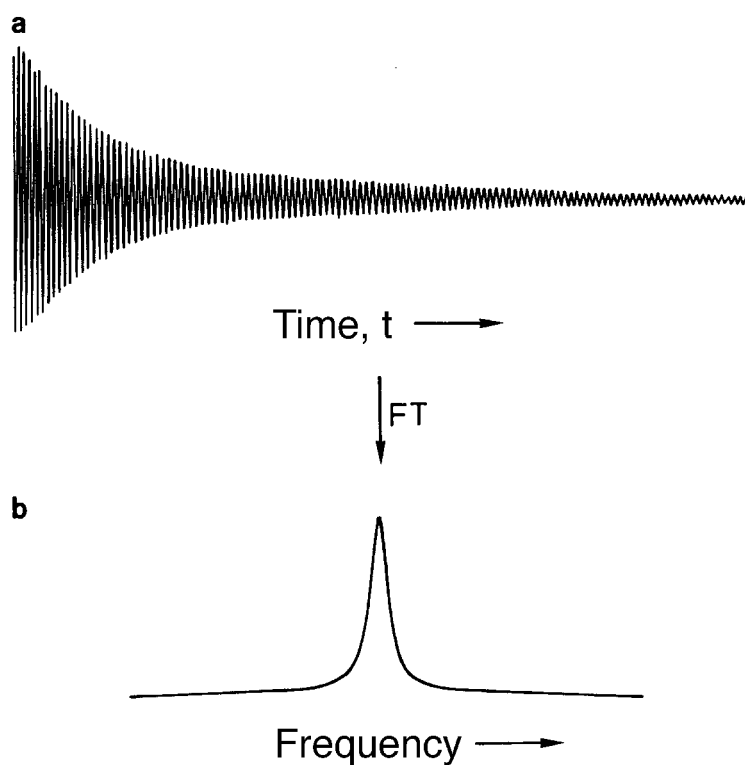
The free induction decay (FID) is the decline of the transverse magnetization M_{xy} after a high frequency pulse. For example, we will consider the situation after a 90 ° pulse. The transverse magnetization is maximal in y -direction, and the value of M'_{xy} is the intensity of the NMR

signal. After the B_1 pulse the phase coherence of the spins will decline with the relaxation time T_2 :

$$\frac{dM_x}{dt} = -\frac{1}{T_2} M_x; \frac{dM_y}{dt} = -\frac{1}{T_2} M_y; M_{xy} = M_{xy0} \exp\left(-\frac{1}{T_2}\right) \quad \text{Eq. 6.26}$$



FID after a 90° pulse



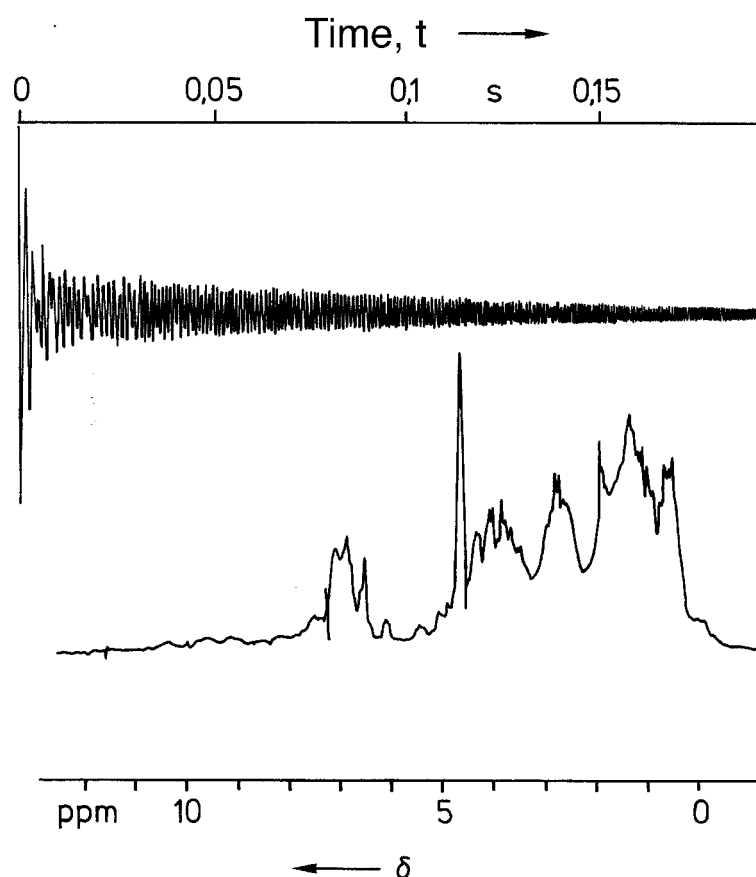
Free induction decay (FID) after a 90° pulse and Fourier transformation.

Figure 6.22 Free induction decay and Fourier transformation after a 90° pulse.

in an ideal homogeneous magnetic field B_0 . In an NMR experiment the field B_0 is not ideal and there are slight inhomogeneities such that the free induction decay is given by

$$\frac{dM_y}{dt} = -\frac{M_y}{T_2^*} \quad \text{Eq. 6.27}$$

The relaxation time T_2^* contains both the contribution of the transverse relaxation and the field inhomogeneities of the magnetic field B_0 .

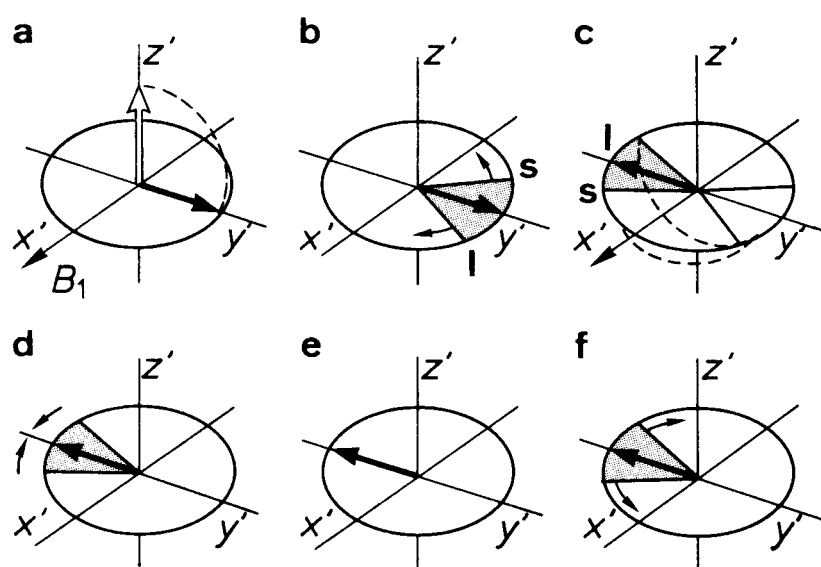


FT ^1H -NMR spectrum of basic pancreatic trypsin inhibitor at 100 MHz. 100 FIDs were accumulated after 90° pulses. The spectrum is then obtained by Fourier transformation.

Figure 6.23 ^1H NMR time and frequency domain spectra of bovine pancreatic trypsin inhibitor.

The decay function of the transverse magnetization that is depicted in Figure 6.22 is generated only when the pulse frequency is identical to the Larmor frequency of the nucleus, i.e. this function is only observed at one particular chemical shift. The advantage of the FT-NMR is the concurrent observation of the whole spectrum by a high frequency pulse that contains all relevant frequencies. This means that the pulse also contains “off resonance” frequencies.

The resonance frequency and the off resonance frequencies will interfere and the recorded signal will have the line shape of superimposed decaying sine functions. The typical differences between the Larmor and the pulse frequency are approx. 50 Hz. The information that is contained in the time domain in case of resonance and in case of non resonance can be measured and converted from the time domain to the frequency domain by Fourier transform spectroscopy. For protons with different chemical shifts there will always also be the case of non-resonance such that the time domain spectrum is composed of complex superpositions of absorbance signals. After accumulating numerous FIDs, the frequency domain spectrum is calculated (for an example see Figure 6.23).



Spin-echo experiment after Hahn. **a** A 90° pulse in the direction of the x' -axis changes the magnetization from the z -direction to the y' -direction. **b** Due to magnetic field inhomogeneities, some spins are precessing faster (s) than others (l) with respect to the mean value on the y' -axis. The coherence between the spins is lost after some time and M_y becomes smaller. **c** At time t a 180° pulse is performed in x' -direction. The magnetization rotates 180° around the x' -axis. **d** After the 180° pulse, the magnetization still precesses in the $x'y'$ -plane, but the l spins are now ahead of the mean-value and the s spins are behind. The phase coherence is re-established, i.e. M_y increases. **e** 2τ after the 180° pulse, all spins are in phase on average. The detected magnetization at $t=2\tau$, the spin-echo, is of the same value as after the 90° pulse and at $t>2\tau$ the spins again lose their phase coherence and the process can be repeated.

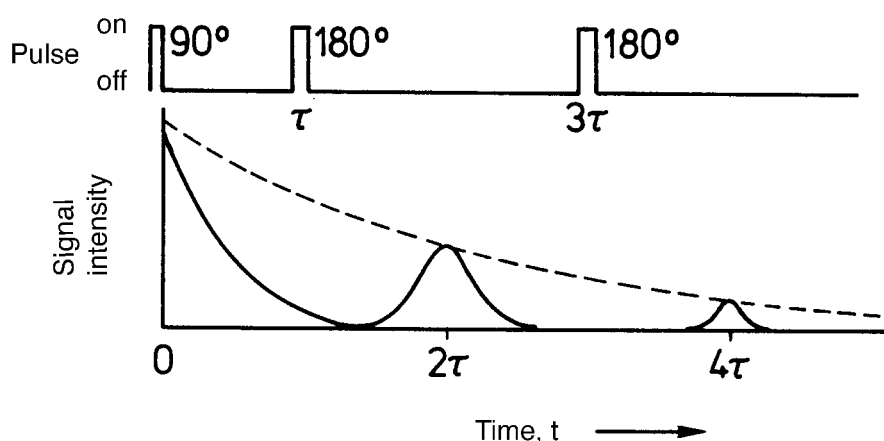
Figure 6.24 Spin-echo experiment after Hahn to determine the T_2 relaxation time.

6.8.3 Spin-echo method to determine the spin-spin relaxation time T_2 .

In a simple experiment, the spin-spin relaxation time can only be measured, if T_2 is small

compared to the contribution of magnetic field inhomogeneities. Otherwise, a value $T_2^* < T_2$ is determined. An experimental method to avoid the problem of field inhomogeneities is the so called spin-echo method by Hahn. This is a multiple pulse method, in which a 90° pulse is followed by a 180° pulse after a delay time τ .

In absence of a spin-spin relaxation, the sequence $90^\circ\text{--}\tau\text{--}180^\circ$ eliminates the different precession frequencies due to magnetic field inhomogeneities. If we consider the spin-spin relaxation, then the transverse magnetization M_{xy} will decline within the time 2τ , because of spin-spin relaxation leading to an equal distribution of the spins on the cone of precession. This effect cannot be avoided by the pulse sequence, such that the spin-echo (M_y'), the signal intensity, is smaller after 2τ and will further decline after every further pulse sequence. From a logarithmic plot of the echo amplitude as a function of the delay time t , the spin-spin relaxation can be determined from a series of $90^\circ\text{--}\tau\text{--}180^\circ$ pulses (Figure 6.25).



Pulse sequence $90^\circ\text{--}\tau\text{--}180^\circ$ and spin-echo after 2τ and 4τ

Figure 6.25 Spin-echo intensity as a function of the delay time in a $90^\circ\text{--}t\text{--}180^\circ$ pulse sequence.

6.8.4 Determination of T_1 .

The spin-lattice relaxation time can also be determined by multiple pulse experiments. Two methods, the progressive saturation and the inversion recovery method shall be described.

6.8.4.1 Progressive saturation.

By 5 to 10 90° pulses, a dynamic equilibrium between absorption and relaxation is obtained. After a time τ , the intensity of the NMR signal is measured. If the longitudinal magnetization is not yet fully restored, the intensity of the NMR line will be lower than the maximum value. The intensity of the line (A) will increase with τ up to its maximal value A_∞ . A plot of

$(A_{\infty} - A_t)$ as a function of the delay time will show a linear dependence with a slope of $-1/T_1$ (Figure 6.26).

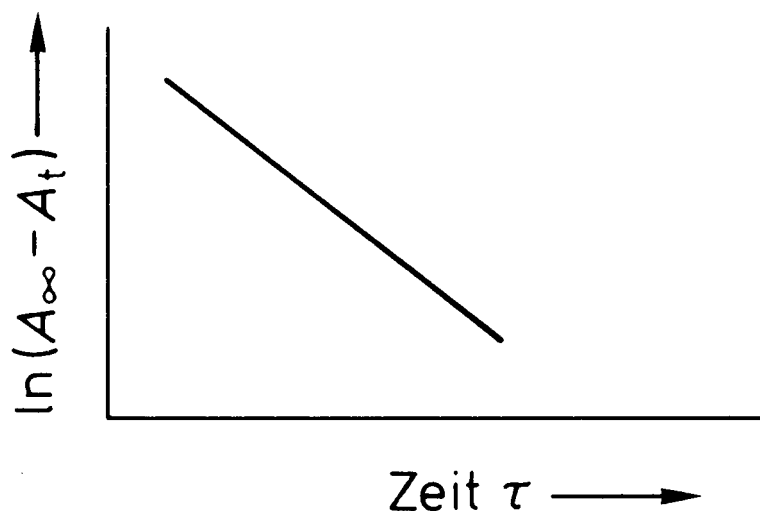
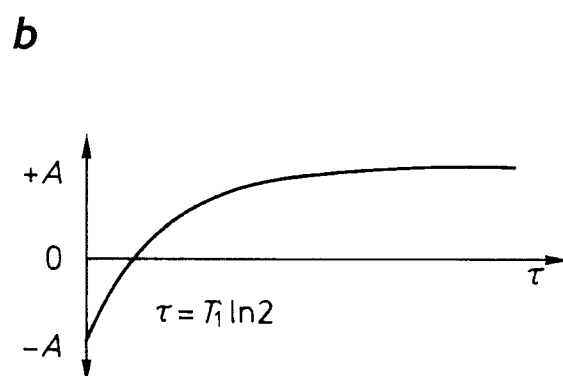
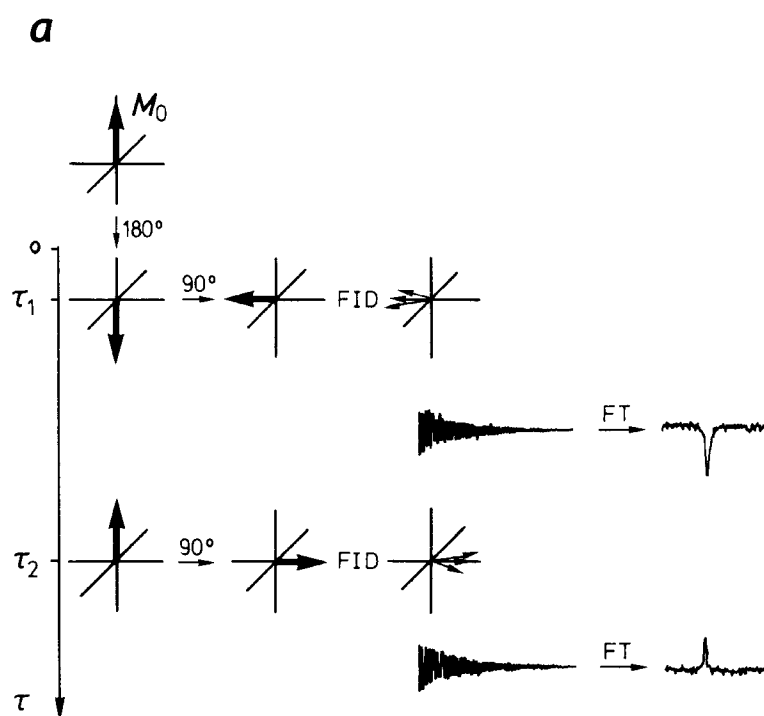


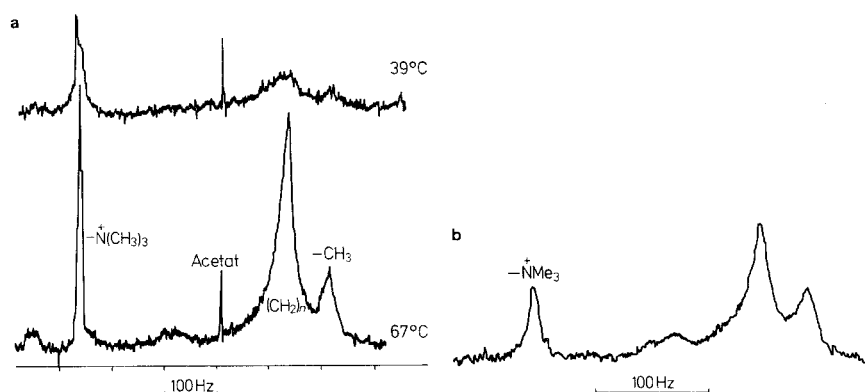
Figure 6.26 Determination of T_1 by progressive saturation. The intensity of the NMR line increases with the time t to its maximum value A_{∞} .

6.8.4.2 Inversion recovery method.

This method works with a $180^\circ - \tau - 90^\circ$ pulse sequence. The 180° pulse causes an inverted magnetization in z -direction. After a delay time t , a transverse magnetization is generated by a 90° pulse and the NMR signal is measured by recording of the free induction decay. With very short times, the magnetization is still pointing to the antiparallel direction and a negative signal is obtained. Only after longer times τ , a positive signal is obtained due to spin-lattice relaxation. In between we observe no signal with $M_z = 0$ and $M_y' = 0$ at $\tau = T_1$. To obtain T_1 , the signal must be recorded as a function of the time τ (up to 100 s). The intensity increases from $-A$ to $+A$.

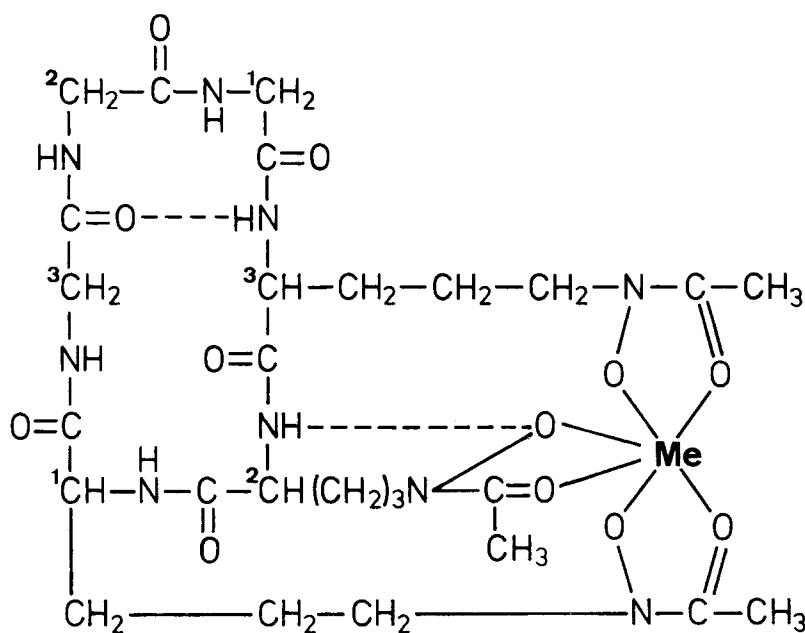


Inversion recovery method to determine the longitudinal relaxation time (spin-lattice relaxation time) T_1 . If the 90° pulse is performed within the time in which M_z is inverted, a negative signal is obtained.

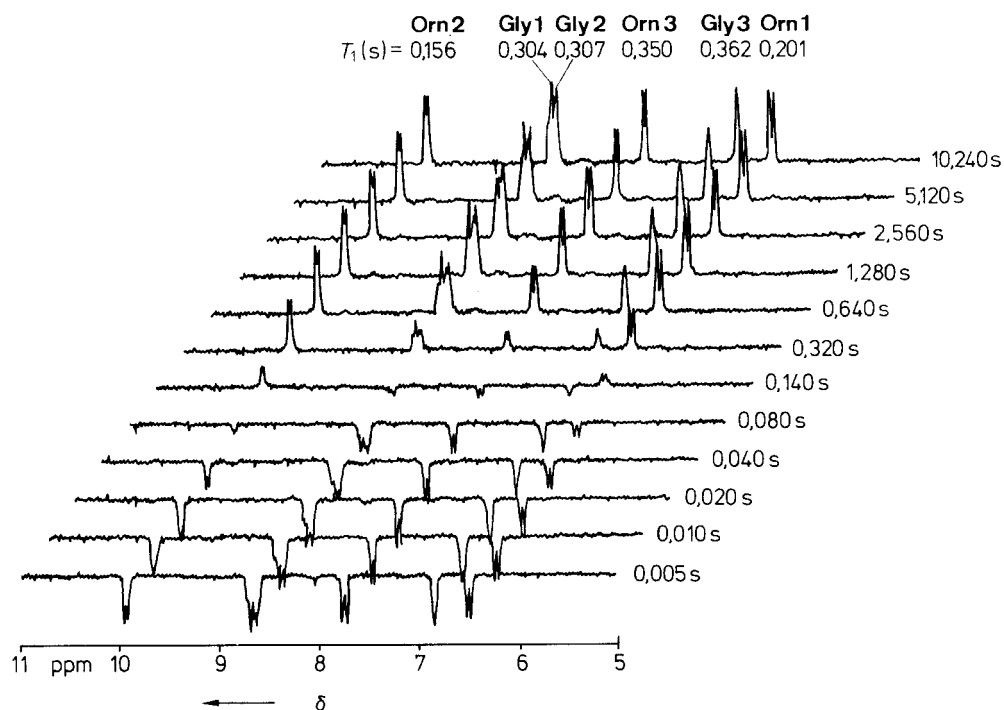


a ^1H -NMR spectra of dipalmitoylphosphatidylcholine in aqueous dispersions below and above the phase transition temperature of the lipid.

b ^1H -NMR spectrum of vesicles prepared from membranes of the sarcoplasmic reticulum.



Ferrichrome Iron. The metal ion ion is complexed by a chelate ligand that consists of three glycines and three δ -N-acetyl- δ -N-hydroxyornithines.



Partially relaxed FT ^1H -NMR spectra of the amide protons of Ferrichrome (Al^{3+}) in deuterated DMSO. The delay times after the 180° pulse in a 180° -t- 90° pulse sequence are given on the right hand side. The T_1 relaxation times are given at the band assignments.

6.9. NMR Spectroscopy *in vivo*.

In the past 5 decades, the NMR spectroscopy has helped to clarify many chemical and biochemical questions, especially in the investigation of chemical reactions in a test tube. A combined approach of ^{13}C -NMR and ^1H -NMR techniques was mostly used. In the last two decades, researchers developed the “*in vivo*” NMR spectroscopy, the magnetic resonance spectroscopy of living organisms. The NMR techniques then became a valuable tool for biologists and physicians.

Which new insights can be obtained from NMR spectroscopy “*in vivo*”? How is it possible to obtain information on a large living object, especially given the fact that NMR-spectra of simple molecules are often already very complex? How is it possible to measure such large samples like humans?

In “*in vivo*” NMR experiments, researchers are interested to study chemical reactions in living cells without interferences from outside. The biggest problems that are faced in “*in vivo*” NMR spectroscopy lie in the magnitude of different molecules and their small concentrations in which they are present in the body. How is it possible to obtain signals and how can these be assigned to defined molecules in the body?

NMR spectrometers for “*in vivo*” investigation consist of large magnets which can completely enclose a human body. Technically, the construction of such an NMR spectrometers is solved and these spectrometers are already used in routine experiments. Local NMR experiments at specific body parts are performed with “surface coils”, that are adjusted to the surface of the body part under investigation and serve as senders of the high frequency pulse. All other electronic parts are those of a conventional NMR spectrometer. The information that *in vivo* NMR spectrometers give are chemical shifts and intensities of resonance lines. Only rarely coupling constants are obtained since the resolution of the spectrometer is usually not sufficient to resolve the splitting of the signal.

6.9.1 ^{31}P -NMR spectroscopy *in vivo*.

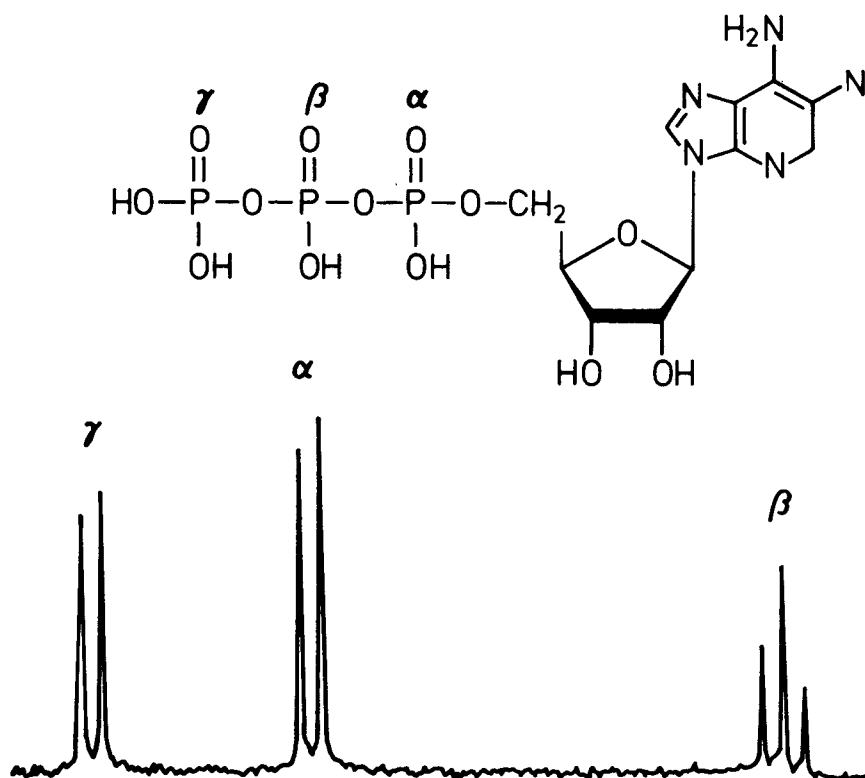
There are several reasons why the nucleus ^{31}P is suitable for *in vivo* NMR spectroscopy.

- organic compounds containing phosphorous are key components in the bioenergetics of living organisms.
- the number of different bioorganic compounds that contain phosphorous is limited.

- ^{31}P resonances can be well resolved. ^{31}P nuclei have a large magnetic moment and the natural abundance of ^{31}P is almost 100%. In addition, the concentration of phosphorous is relatively high in many organs, such that one can obtain spectra in a few seconds.

A large part of in vivo applications of NMR methods deal with the investigation of the bioenergetics of the muscle and for medical reasons, investigations of the heart muscle. Investigations of the heart muscle are aggravated because of the motion of the heart. The observation pulses therefore must be triggered to the beat frequency of the heart.

Before we consider a simple example, that of the ^{31}P -NMR spectrum of the forearm, we have to determine which compounds that contain phosphorous are generally found in the cell. This is relatively straightforward to answer, because the biochemical reactions in energy metabolism of the muscle have been investigated intensively. Phosphate is transferred from



^{31}P -NMR spectrum of adenosine triphosphate at 49.58 MHz with phosphorous acid as a reference. The peak assignment is according to the annotation of the structure above. The nuclei are coupled and Doublets and Triplets are observed.

Figure 6.27 ^{31}P -NMR spectrum of adenosine triphosphate.

creatin phosphate (PCr) to adenosine diphosphate (ADP) to form adenosine triphosphate (ATP) and creatin (Cr). The ^{31}P -NMR spectrum of ATP is shown in Figure 6.27. The nuclear spin of ^{16}O is zero and therefore the ^{31}P -NMR lines are split only by $2J$ coupling between the phosphorous nuclei. P_α and P_γ are split into a doublet while P_β is split into a triplet, due to the coupling with two neighbor nuclei. The resonance lines of ATP are clearly observed by their chemical shifts and by their relative signal intensities also in the spectrum of a human forearm Figure 6.28. This spectrum contains two additional resonance lines, that of

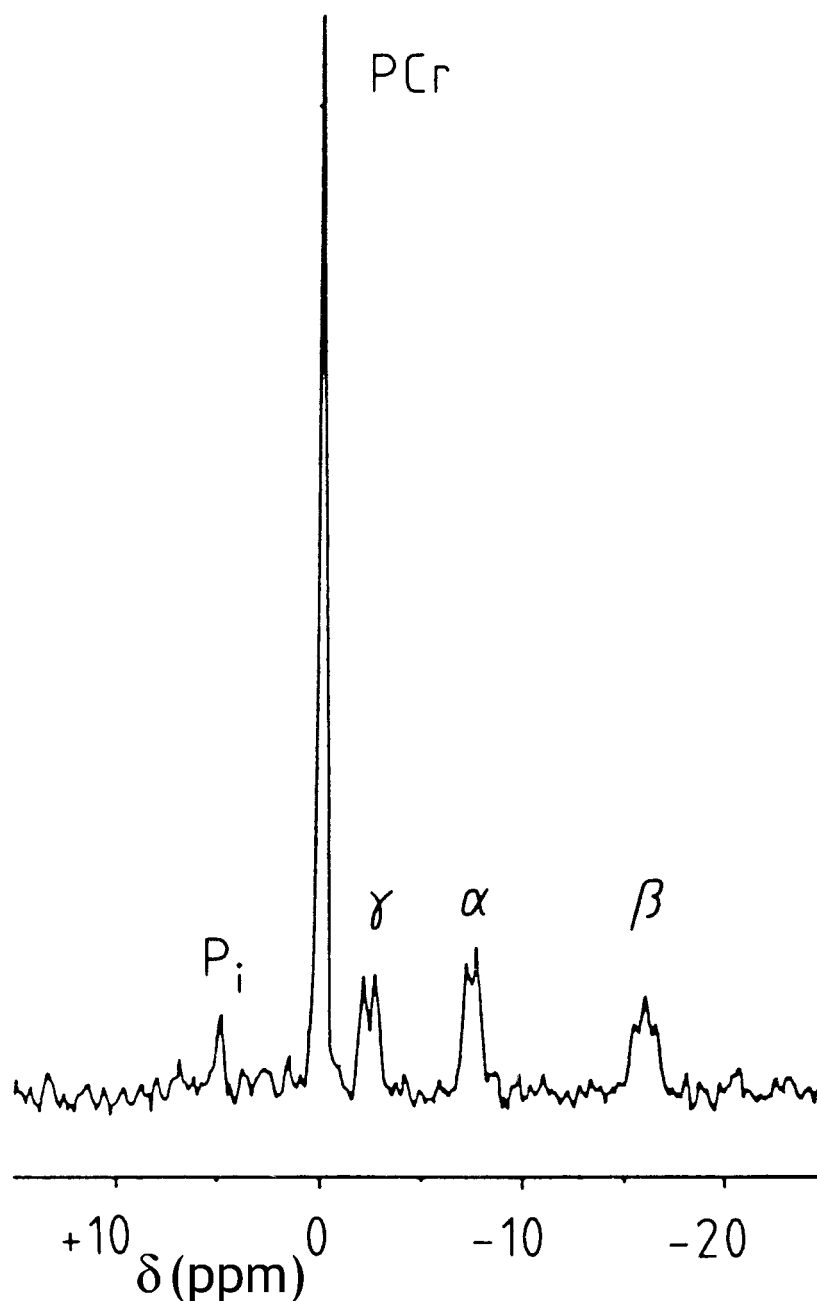
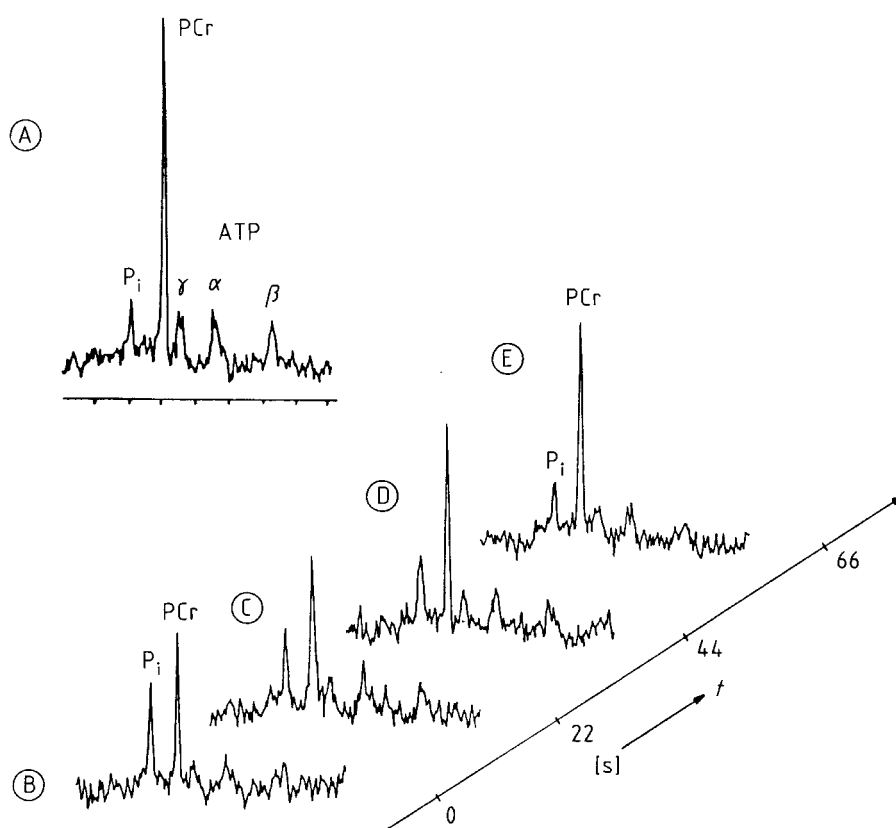


Figure 6.28 ^{31}P -NMR spectrum of a human forearm.

creatine phosphate (PCr) and that of free phosphate (P_i). Signals of ADP, that is also present, are located below the signals α and γ of ATP. The signals of other phosphor organic compounds disappear in the background noise of the spectrum. The signal from the human forearm is dependent on the condition. It will be different if the arm has rested or was used in hard work

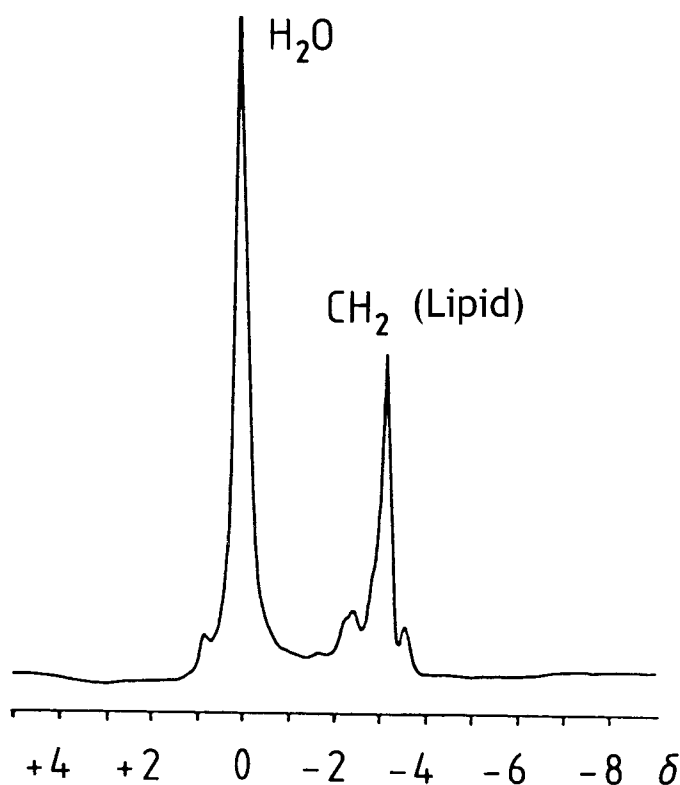


Sequence of ^{31}P -NMR spectra of a human forearm after 10 min of hard exercise. 40 MHz spectra were recorded with a surface coil of a diameter of 4 cm. 10 scans were accumulated per spectrum. The spin system was allowed to relax for 2 s. The total time required to record one spectrum was 22 s. The control spectrum (A) was recorded before the exercise. The patient then had to squeeze a ball outside the magnet until he was unable to continue. The spectra B to E were recorded after completion of the exercise.

Figure 6.29 Spectra of the human forearm as a function of physical stress.

or exercise. Figure 6.29 displays the spectrum of the forearm at rest (A) and at different times after exposure to physical stress. Immediately after performing work, the signal of PCr is reduced, while the spectrum of the inorganic phosphate is strongly increased. After 66 s the original concentrations of all compounds are restored and the spectrum corresponds to the spectrum in the resting condition. The ratio of the organic phosphorous compounds has changed relative to the relaxed state after the forearm was exposed to a physical stress

condition. More dramatic effects are found, if the muscle had a reduced supply of oxygen over a longer time (ischemia). This is possible, if the bloodstream is reduced by a constriction of the blood vessels. The most prominent example is the myocardial infarction (ger.: Herzinfarkt).



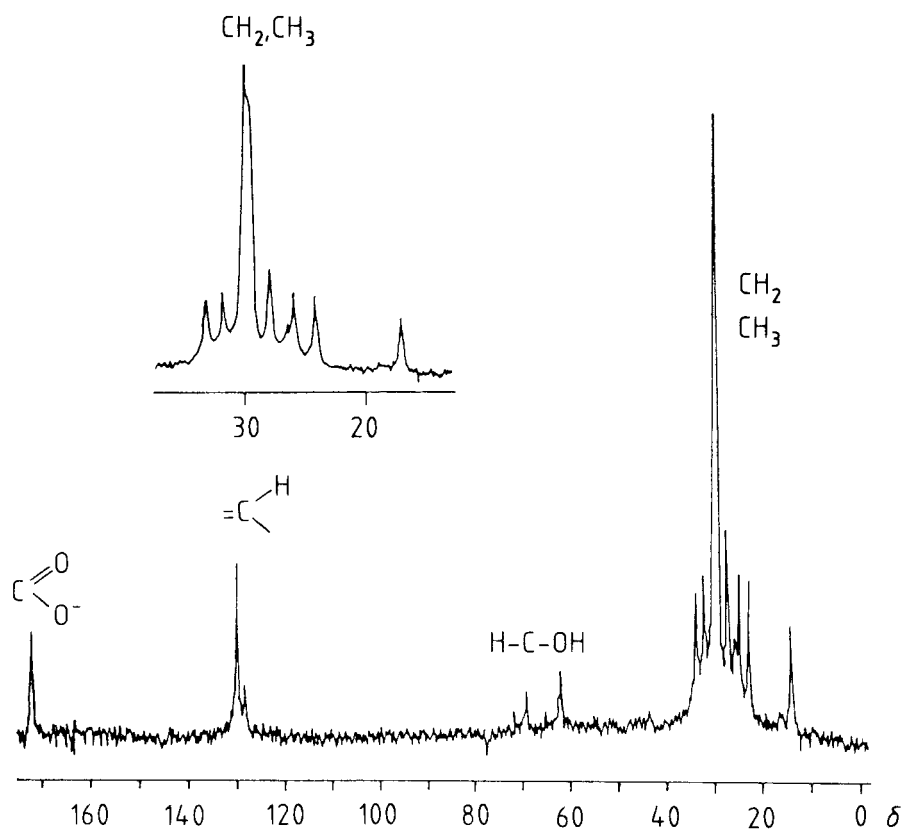
100 MHz ^1H -NMR spectrum of the human forearm. The major signals are that of water and that of methylene protons of lipids. The δ -scale shown here is based on water as the standard. 2 scans were averaged. Acquisition time: 5 s.

Figure 6.30 ^1H -NMR spectrum of the human forearm.

6.9.1.1 ^{13}C and ^1H NMR spectroscopy in vivo.

Problems that have to be overcome for in vivo applications of ^{13}C and ^1H NMR spectroscopy are

- a large variety of mixed organic compounds are present, often of highly complex molecules.
- organic substances in the body are often present at very low concentrations.
- the solvent water
- small chemical shifts in ^1H -NMR spectroscopy.
- broad lines as a result of field inhomogeneities.



25 MHz broad-band decoupled ^{13}C -NMR-spectrum of the human forearm. 500 scans were averaged (acquisition time: 10 min). Signals must be assigned to the carbon atoms of lipids (glycerol and fatty acids).

Figure 6.31 ^{13}C -NMR spectrum of the human forearm

Figure 6.30 shows the ^1H -NMR spectrum of the human forearm. Two signals dominate the spectrum. The first is a strong absorption line of water, that is also used as a reference in the chemical shifts of other molecules in *in vivo* ^1H -NMR spectroscopy. The second resonance line is that of the methylene group that is present in the lipids of the body. Most other signals are of negligible intensity.

More information can be obtained from ^{13}C -NMR spectroscopy. Figure 6.31 shows the ^{13}C -NMR spectrum of a human forearm. Since the chemical shifts of ^{13}C are relatively large, the signals of the lipids in the forearm can be distinguished. The carboxy-groups have the largest chemical shift ($\delta=172$). Chemical shifts of $\delta=130$ for the carbons that participate in a double bond, $\delta=60\text{--}75$ for the carbon atoms of the glycerol backbone, and $\delta=10\text{--}35$ for the carbons of the aliphatic fatty acids signals are observed. For the spectrum shown in Figure 6.31 it was necessary to accumulate 500 scans and the total acquisition time for the spectrum was 10 min. In modern investigations, organic compounds enriched in ^{13}C are used.

6.9.1.2 Magnetic resonance tomography.

Because of their high sensitivity, the *in vivo* NMR spectroscopy of the protons was developed further. Since living systems are always containing water, the distribution of water in the organism was investigated first. For small volume elements that contain lots of water, stronger signal intensities were expected than for those small volume elements that contain relatively little water. The problem therefore was, to develop a method to measure the distribution of water in the body by ^1H -NMR spectroscopy. Three technical problems had to be addressed:

- The size of the magnet. For example, if the water distribution in a human head must be determined then the magnet has to have an opening large enough for the head. In conventional high resolution NMR spectrometers, this opening is only a few cm wide. This problem is solved nowadays and electromagnets with a magnetic field strength of up to 0.3 T are used. Sometimes even cryomagnets with a field strength up to 3 T are part of *in vivo* NMR tomographs.
- The second problem is also a technical difficulty, that must be overcome. The resonance condition must be fulfilled to obtain an NMR signal and this is difficult for a heterogeneous large object like a human head. In NMR tomography, only small volume elements are analyzed in which a local field B_0 is generated. In these volume elements (*voxels*) the resonance condition is met in the inside but not in the outside or in their surface regions. For a fine resolution these voxels need to be small. The disadvantage of smaller voxels is the reduced signal intensity due to a smaller number of ^1H nuclei. The data acquisition time is therefore the longer the smaller the volume element. With mice and other small animals the highest spatial

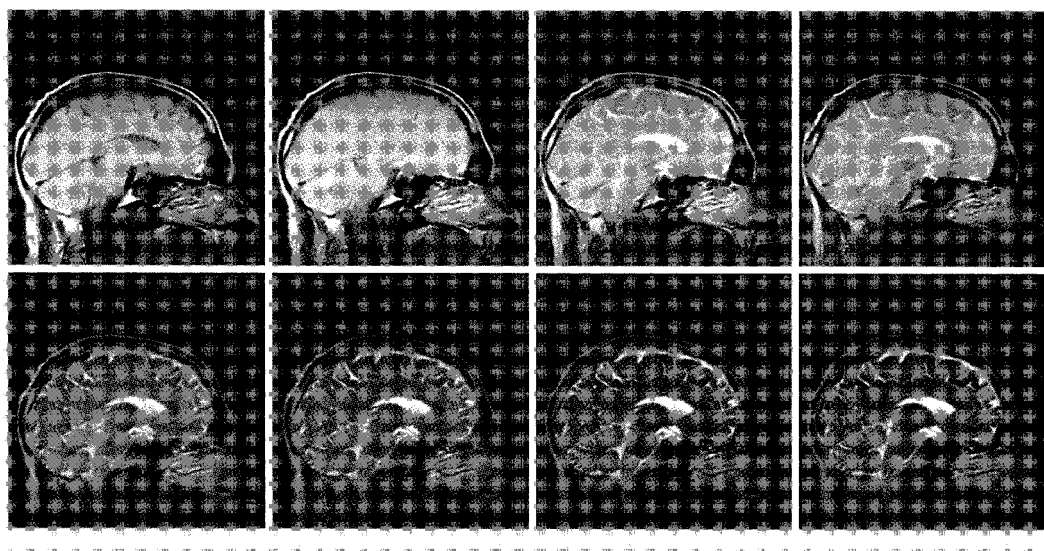
resolution corresponds to a volume element of $10 \times 10 \times 100 \text{ } \mu\text{m}$. The imaging with such a high resolution is termed NMR microscopy, although the resolution of optical spectroscopy is not yet matched by NMR microscopes. The selection of the volume element is achieved by magnetic fields that have a variable gradient of the magnetic field strength. With these magnetic field gradients, slices of different magnetic field strengths are formed through the object. These slices are subsequently divided into small volumes. Usually, these slices are about 7 mm thick and single volume elements are rods of 1 mm diameter and a length of about 7 mm. To image the complete head, at least 8 such slices are necessary. The result of NMR-absorption of each volume element is registered, stored, and converted into a signal by Fourier transform spectroscopy.

- The third problem is that of imaging (reconstruction of the image from the individual measurements on each voxel). This problem could be solved in analogy to the experiences obtained from x-ray tomography. Today it takes only a few seconds to measure the water distribution in a particular object.

From the medical point of view, the distribution of water is not all that exciting. More interesting became the magnetic resonance tomography by the fact that the relaxation times T_1 and T_2 depend characteristically on the environment of the water, i.e. on the tissue to which the water is bound. The images that are required therefore should reflect not only the density distribution of water, but also the changes in the relaxation times T_1 and T_2 .

We have previously discussed how one can obtain the relaxation times T_1 and T_2 by pulse sequences $90^\circ\text{-}\tau\text{-}180^\circ$ (T_2) and $180^\circ\text{-}\tau\text{-}90^\circ$ (T_1). The spin-system relaxes after a 180° pulse with T_1 and the intensity of the signal will be zero after a time $\tau = T_1 \ln(2)$ s. Nuclei with different T_1 time have a different time τ at which the signal intensity becomes zero. This is also true for protons in water, if the T_1 times are not the same because of different environment of the water molecules in different tissues. At any given time t , the images obtained will differ in contrast or color.

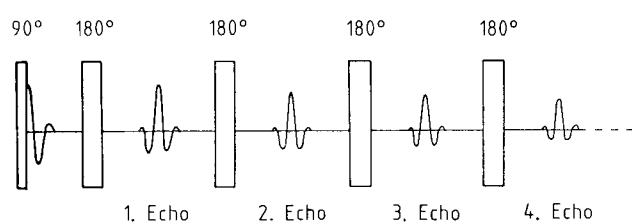
A similar dependence on location of the water is also observed for the spin-spin relaxation time, T_2 . To illustrate the dependence of T_2 on the water density, a spin-echo experiment is performed. The regions of different contrast in the image indicate a distribution of different spin-spin relaxation times. The quality of the images is comparable to that of an x-ray tomogram, and maybe even of better quality in some cases. The NMR experiment has one big advantage: It does not expose the body to damaging x-ray radiation. In this way, the experiments may be repeated without danger. Risks for the human health imposed by magnetic fields or by switching them on and off are currently not known.



Medio-Sagittal cut through the skull. In this experiment, a multiple echo sequence was used in which 8 echos were recorded. Each image of this series corresponds to one of the 8 echos. In each image, the same slice of 7 mm thickness is shown, which was divided into 256×256 matrix elements. For each matrix element, two spectra (FIDs) were accumulated. The total acquisition time for the eight images was about 11 min. (The images were recorded on a NMR tomograph of the Company Bruker, Rheinstetten, Karlsruhe, with a cryo-magnet at a field strength of 1.5 T.)

Figure 6.32 NMR Tomography of a human skull. Each image corresponds to a spin-echo.

Figure 6.32 shows 8 images of a medio-sagittal slice through a human skull. A slice of 7 mm thickness was recorded. The pulse sequence that was used in this experiment is shown in

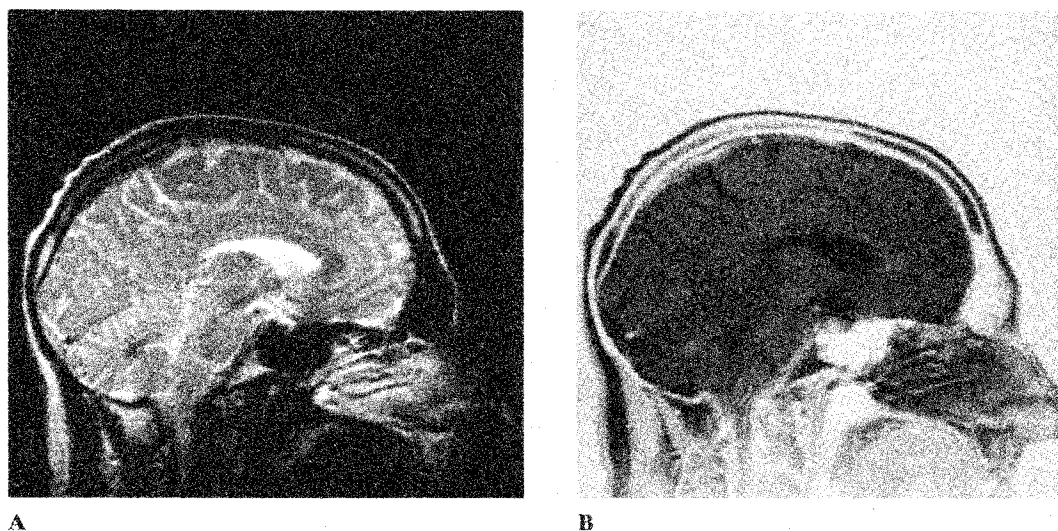


Multiple echo sequence after Carr-Purcell-Meiboom-Gill. The first 90° pulse prepares the spin system, the following 180° pulses generate the echos. For skull imaging, 8 such echos were recorded.

Figure 6.33 Multi echo sequence after Carr-Purcell-Meiboom-Gill. The first 90° pulse prepares the spin-system, the following 180° pulses generate the echoes.

Figure 6.33. Each of the 8 images shown in Figure 6.32 corresponds to one of eight echoes. The first image very closely corresponds to the effective water distribution in the skull (light contrast corresponds to water rich regions, dark contrast to relatively little water) The series of images shows that the contrast changes from echo to echo and the contours become

better visible. The changes in contrast are a direct consequence of the different spin-spin relaxation times T_2 in the different types of tissues of the brain. It is also visible that the overall brightness of the image is reduced from the first to the last image due to relaxation. Between excitation and the 8th echo a time of 0.3 s has passed. The complete time for the recording of the 8 echoes was 11 min.

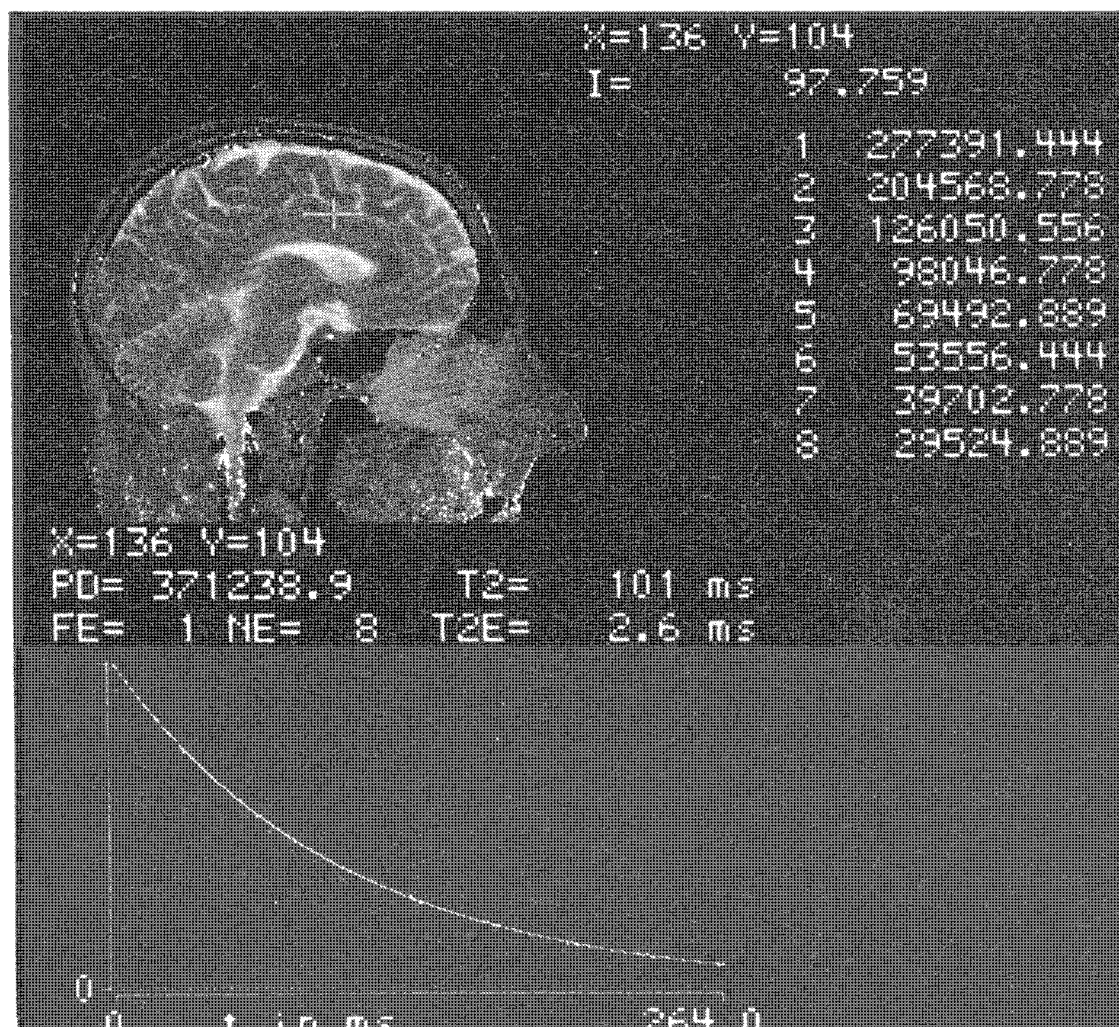


Medio-Sagittal slice of a skull. In A, eight echo images are superimposed. B is the negative of A.

Figure 6.34 A sum of the eight images shown in Figure 6.32 . B Negative of A, showing the bones in white color.

In Figure 6.34 A, the sum of the 8 echoes shown in Figure 6.32 is shown. This representation will give images that are very rich in contrast. In Figure 6.34 B the negative is shown, that is also computer generated. In the negative image the bones have white color. In Figure 6.35 , one matrix element (voxel) is marked and the relaxation time T_2 can be obtained from the intensities in each of the eight echoes of Figure 6.32 . It is usually also calculated by the computer ($T_2=101$ ms in this case).

In medical applications it is of importance that the relaxation times T_1 and T_2 are very different between healthy and unhealthy tissue.

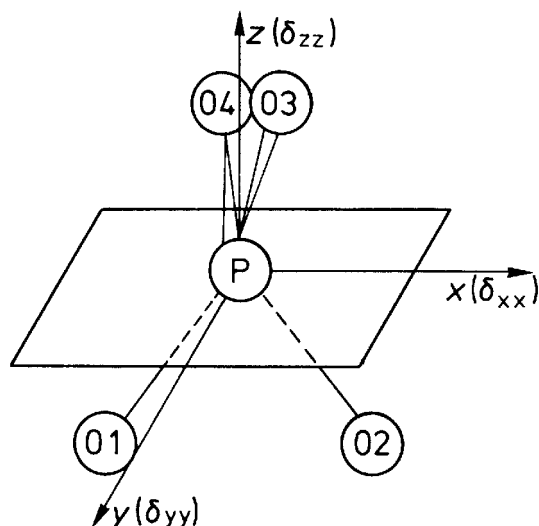


Estimation of the spin-spin relaxation times T_2 . The diagram shows the intensities (signal amplitudes) of the 8 individual echoes for the location indicated by the cross. By moving the cross over the image, the spin-spin relaxation time can be obtained at any point of the image.

Figure 6.35 Estimation of the spin-spin relaxation time at each position within the slice through the skull of Figure 6.32 .

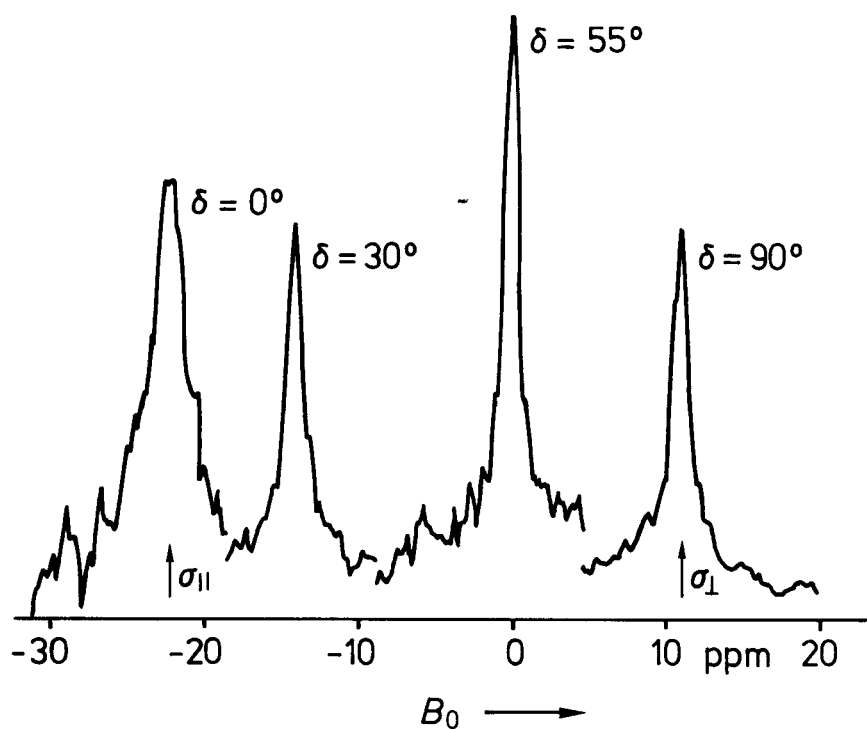
6.9.2 Chemical shift anisotropy.

The NMR resonances depend on the chemical environment of a nucleus. This environment is different in each direction in space. However, in solution NMR experiments, the molecules usually tumble and rotate rapidly in space, a motion which is usually faster than the time required for NMR transitions. The differences in the chemical environment along each coordinate in space become only apparent, if molecules are aligned with a preferred direction in space (relative to the magnetic field B_0). An example for such an alignment are the lipid molecules in a lipid bilayer which is supported for example by a glass plate (or by a germanium



Anisotropy of the chemical environment and the chemical shift of ^{31}P in the phosphate group of a phospholipid. O3 and O4 are the free oxygens and O2 and O1 the oxygens in the ester bonds. [After Seelig, J. (1978), *Biochim. Biophys. Acta* 515, 105].

Figure 6.36 Anisotropy of the chemical shifts of ^{31}P in the phosphate group of a phospholipid.

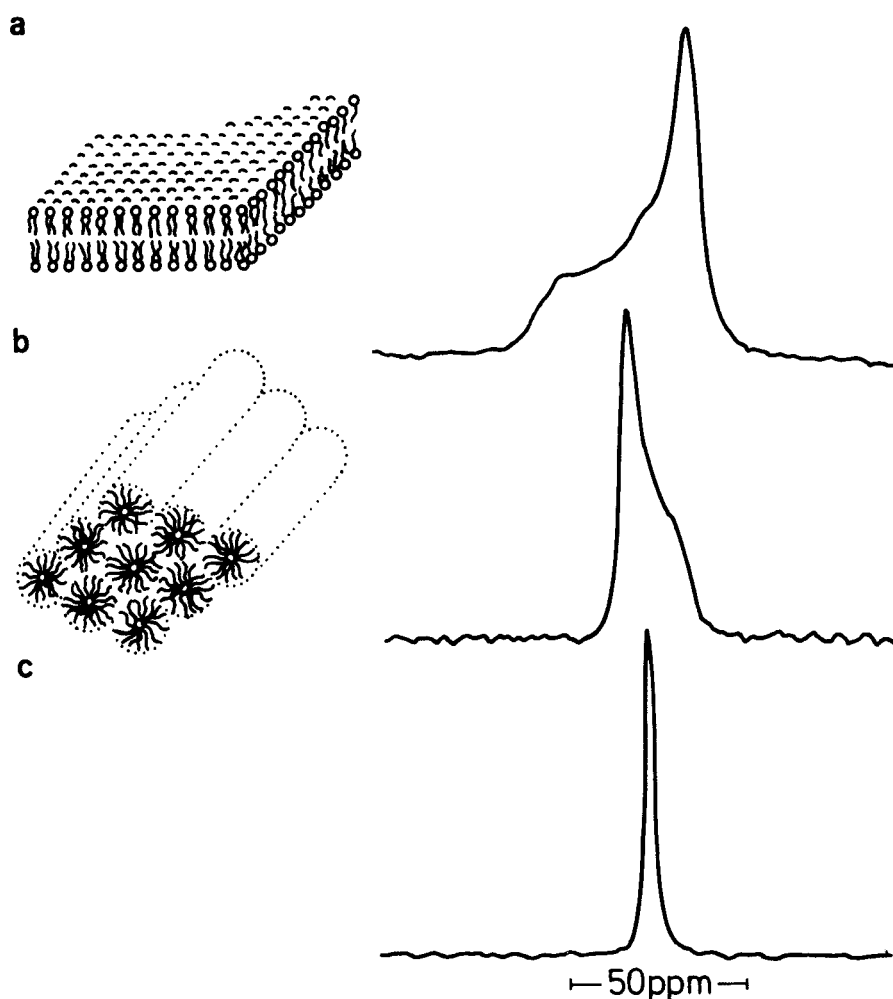


^{31}P -NMR spectra of oriented samples of a phospholipid in planar lipid multilayers. δ is the angle between the magnetic field B_0 and the membrane normal. [After Seelig, J. Gally, H. (1976), *Biochemistry* 15, 5199]

Figure 6.37 Chemical shifts of ^{31}P in spatially oriented phospholipids.

plate). The differences in the chemical environment of the ^{31}P nucleus of the phosphate group are shown in Figure 6.36. If the lipids are oriented with their molecular long axis in x, y, and z-direction (relative to the field B_0 , which is in z-direction), different chemical shifts of the ^{31}P nucleus are observed (which is a consequence of the different electron density of the phosphate group in a phospholipid in each direction in space).

If phospholipid molecules are not oriented in space, there are many cases in which these molecules are still not allowed to tumble and rotate freely. For example, their motions can be restricted by the supramolecular structure of the lipid-bilayer. Thus, phospholipid NMR-spectra are powder pattern spectra that consist of a superposition of the NMR spectra of all molecules with different orientations in space. Such spectra are shown for different lipid phases in Figure 6.38. ^{31}P -NMR spectroscopy is therefore a tool to determine supramolecular structure.



^{31}P -NMR spectra of different phases of hydrated phospholipids.
a phospholipid bilayer, **b** hexagonal phase, **c** isotropic micellar phase. [After Cullis, P.R., Hoppe, M.J. (1978), Nature 271, 672].

Figure 6.38 ^{31}P -NMR spectra of different lipid phases.

6.9.3 Intensity gain by selective population inversion.

The modest sensitivity of the resonances of many nuclei is a major problem in NMR spectroscopy. The reason is the small difference in the population of the different energy levels (see section 6.7.1). The peak intensity is proportional to the difference of the populations of the two energy levels, $n_h - n_l$, between which the transition takes place. The ratio of the two populations is given by a Boltzmann relation:

$$\frac{n_h}{n_l} = \exp\left(-\frac{\Delta E}{kT}\right) = \exp\left(-\frac{g_N \beta_N B_0}{kT}\right) = \exp\left(-\frac{g_N \hbar B_0}{kT}\right) \approx 1 - \frac{g_N \hbar B_0}{kT}$$

Eq. 6.28

The difference between these two energy levels is the larger the larger the magnetic field B_0 . This is one of the reasons why the development of new NMR spectrometers aims to increase the magnetic field strength.

Selective Population Inversion Experiment (SPI):
Transfer of Polarization.

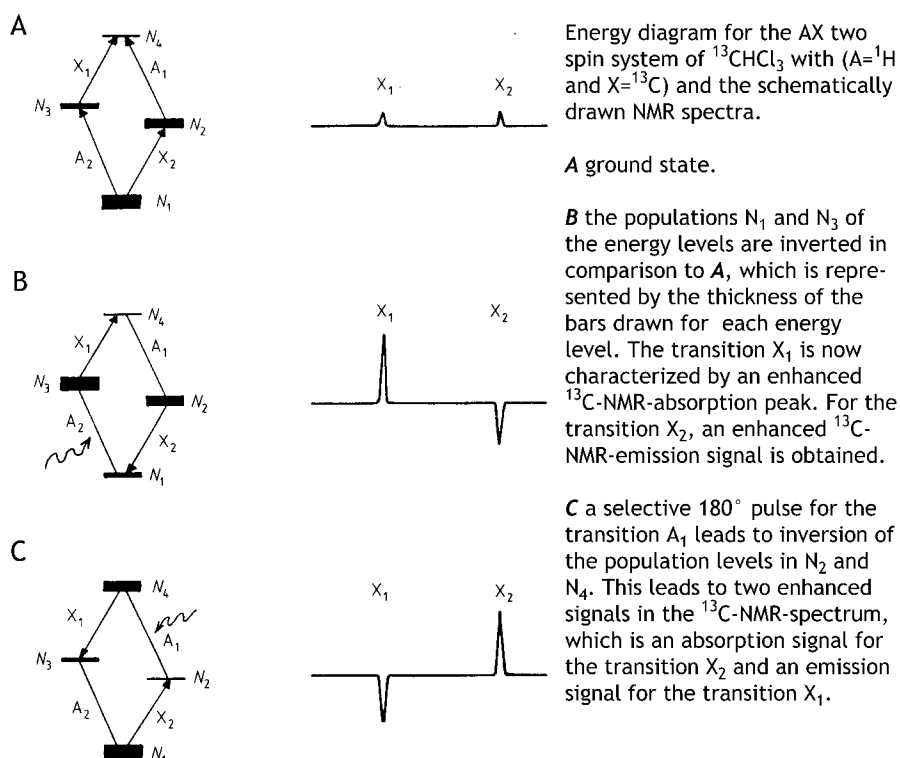


Figure 6.39 Schematic diagram of a polarization transfer experiment, here that of a selective population inversion experiment (SPI).

However, the population difference and thus the signal strength, is also dependent on the magnetogyric ratio γ . Nuclei like ^1H , ^{19}F , and ^{31}P have a large γ and are therefore better detectable than nuclei such as ^{13}C , ^{15}N and ^2H . In addition, the less sensitive nuclei are often also less abundant in nature, which is an additional disadvantage. Special experiments with selective pulses increase the intensity of the NMR absorption peaks. One example of such experiments is the selective population inversion experiment (SPI). To illustrate the experiment, chloroform $^{13}\text{CHCl}_3$ shall be considered. The ^{13}C NMR spectrum consists of a doublet, due to coupling between the spin of the ^{13}C (spin 1/2) and the spin of the ^1H ($J(\text{C},\text{H}) = 209 \text{ Hz}$). Would the ^1H broad band decoupler be turned on, only one signal would be observed. The spectrum is depicted in Figure 6.39A. The transitions of the protons A_1 and A_2 are observed in the ^1H -NMR spectrum as satellites of the main signal, if normal Chloroform (containing mostly ^{12}C) is used. The populations N_1 to N_4 of the 4 possible different energy levels is again given by Boltzmann distributions (Eq. 6.28). Eq. 6.28 allows to deduce the relative populations of the energy levels N_1 to N_4 , and therefore the signal intensities of the corresponding transitions. To estimate the relative populations, one must consider that the magnetogyric ratio of ^1H is about four times larger than that of ^{13}C . The differences between N_1 and N_2 and also between N_3 and N_4 are small, because they are determined by the magnetogyric ratio of ^{13}C . In contrast, the differences between N_1 and N_3 and between N_2 and N_4 are larger, because these differences are determined by the magnetogyric ratio of the proton. Thus the energy levels N_1 and N_2 are stronger populated than the energy levels N_3 and N_4 . A selective 180° pulse to induce a transition A_2 will invert the population ratio between levels 1 and 3. Now, N_3 is larger than N_1 , Figure 6.39 B. For the transitions X_1 and X_2 , the situation has now changed: the intensity of the NMR peak corresponding to the transition X_1 will now increase, because the difference between the populations of N_3 and N_4 was increased due to the transfer of magnetization (polarization). For the transition X_2 we now have an increased difference between N_2 and N_1 (with $N_2 > N_1$!). The signal intensity is also increased for this transition, but an emission is observed. Similar considerations may be made for a selective 180° pulse to induce a transition A_2 . Such a pulse inverts the populations of levels 2 and 4. For X_1 an increased emission and for X_2 an increased absorption signal are observed (Figure 6.39 C). The signal amplification depends on the ratio of the magnetogyric ratios of the nuclei:

$$1 + \frac{\gamma_A}{\gamma_X} \quad \text{Eq. 6.29}$$

$$\text{and} \quad 1 - \frac{\gamma_A}{\gamma_X} \quad \text{Eq. 6.30}$$

In case of chloroform ($\gamma(^1\text{H}) / \gamma(^{13}\text{C}) = 4$), the signal intensity is 5 times larger than normal and the emission signal is 3 times larger. Because of the selective inversion of the population ratios, this method is called selective population inversion experiment (SPI). The selective population inversion experiment is an example for polarization transfer. Today, the SPI experiment has been superseded by other techniques, i.e. INEPT (insensitive nuclei enhanced by polarization transfer) and DEPT (distortionless enhancement by polarization transfer) experiments.

6.10. 2-dimensional NMR spectroscopy.

In conventional 1-dimensional NMR spectroscopy signal intensities are given as a function of a chemical shift, i.e. ppm of magnetic field or frequency units. In other words the signal is dependent on the position along one axis. In two-dimensional NMR spectroscopy signals are obtained in dependence of two frequencies and the signal intensity is then plotted in the third dimension. Different kinds of 2-dimensional NMR spectra are distinguished. For example, if chemical shifts are represented by one axis and coupling constants are represented on the second axis the spectra are called 2-dimensional J-resolved NMR spectra. However, if chemical shifts are plotted on both axes, the spectra are called correlated 2-dimensional NMR spectra. Examples for the latter are spectra with ^1H chemical shifts on both axes, (H, H)-COSY, or spectra with ^1H chemical shifts on one axis and ^{13}C chemical shifts on the other axis, (H,C)-COSY. Some different types of two dimensional NMR methods are listed in Table 4.

Experiment	Nucleus	Information
Heteronuclear J-coupled ^{13}C NMR	^{13}C	C,H coupling constants
Homonuclear J-coupled ^1H -NMR	^1H	Estimation of chemical shifts in complex spectra
(H,C) -COSY, correlated spectroscopy	^1H , ^{13}C	Assignment of signals in ^1H and ^{13}C -NMR spectra from known signals
(H,H)-COSY, correlated spectroscopy	^1H	Assignment in complex spectra
H-Relayed-(H,C) COSY	^1H , ^{13}C	Assignments of non-scalar coupled nuclei
H-Relayed-(H,H) COSY	^1H	Assignment of non-scalar coupled protons
Inverse (C,H) -COSY	$^{13}\text{C}/^1\text{H}$	Assignments of non-scalar coupled nuclei
Exchange Spectroscopy NOESY	^1H	Proof of exchange, Proof of spatial neighborhood of nuclei
2D-INADEQUATE	^{13}C	Assignment of coupled ^{13}C nuclei

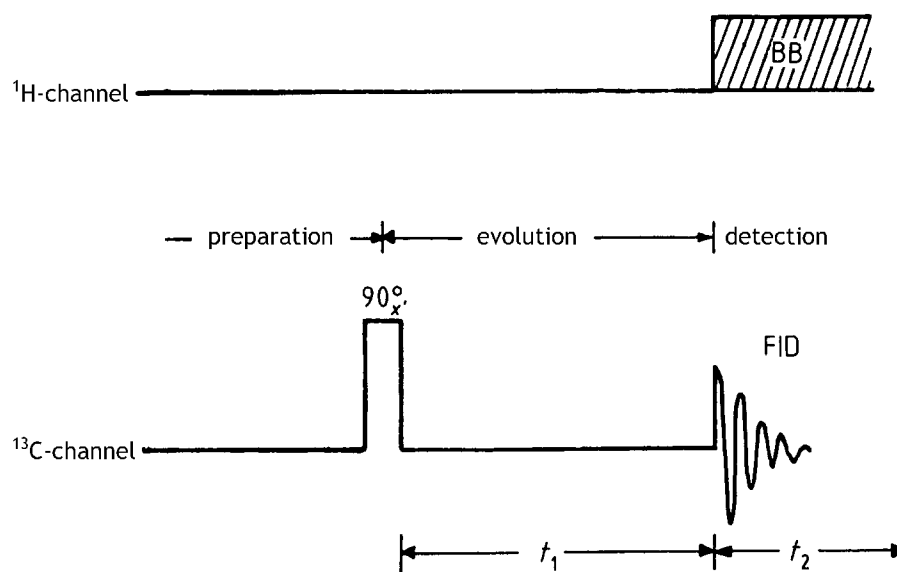
Table 4 Some 2-D NMR experiments

How are such two-dimensional spectra obtained ? Two dimensional spectra require the coupling of nuclear dipoles. It must not necessarily be indirect of J-coupling but can also be through

space coupling of nuclei. The through space coupling that makes use of the nuclear overhauser effect between magnetic dipoles offers an opportunity to study the three-dimensional structure of molecules by nuclear overhauser effect spectroscopy (NOESY). To illustrate some principles of 2-D NMR spectroscopy we will focus on the ^1H and ^{13}C nuclei. In principle the same methods can also be applied to other nuclei.

6.10.1 The 2-dimensional NMR experiment.

In a normal FT-NMR experiment the detection phase immediately follows the excitation of the spin system and the free induction decay is recorded. In 2-D NMR spectroscopy, pulse sequences are more complex and the spin system is “prepared” before the FID is recorded. In addition, the two phases “preparation” and “detection” are extended by a third phase, the evolution phase, in which the spins can couple with each other. The time of this evolution phase is kept variable and two Fourier transformation can now be applied to yield a signal intensity as a function of frequencies that arise from Fourier transformation in the domain of the evolution time and from a Fourier transformation in the domain of the detection time.



Principle of a 2-dimensional ^{13}C -NMR experiment. The time t_1 is a variable. The second variable is the time t_2 . In the time t_1 the spin-system is allowed to “develop”.

Figure 6.40 Principle of a 2-dimensional NMR experiment.

To understand the concept behind the 2-D spectroscopy we perform a simple experiment. We consider a simple 2 spin system AX with A= ^1H and X= ^{13}C . In contrast to a conventional experiment, we introduce a delay time, t_1 , between the preparation of the spin system (which is performed by a 90°_x -pulse, and the acquisition of the free induction decay during a time t_2 . In addition the ^1H -broad band decoupler shall only be switched on during the detection of the free induction decay (Figure 6.40). We assume that we perform n experiments with different t_1 values, starting with $t_1=0$. The time t_1 shall be increased from experiment to experiment by a few ms. The Fourier transformation (FT) of the n interferograms with respect to t_2 results in n different NMR spectra, which consist of a singlet, since during the acquisition of the FID, the ^1H -BB decoupler is turned on. If the broad band decoupler was turned on also during t_1 , the spectra would be all the same except of a decreasing intensity caused by relaxation during t_1 . Since the broad band decoupler was only turned on during the detection phase t_2 , the ^{13}C nuclei and the protons are coupling in the evolution phase. The vector diagrams shown in Figure 6.41 demonstrate how this C,H coupling affects the singlet in the spectrum. After the 90°_x pulse, the macroscopic magnetization of the ^{13}C nuclei is in direction of y' . The magnetization M_C of the ^{13}C nuclei is split into two parts, $M_C^{\text{H}\alpha}$ and $M_C^{\text{H}\beta}$, that correspond to the ^{13}C nuclei in chloroform molecules with a proton of spin α and spin β , respectively. The two vectors, $M_C^{\text{H}\alpha}$ and $M_C^{\text{H}\beta}$, precess with different Larmor frequencies:

$$\begin{aligned} M_C^{\text{H}\alpha} &= \nu_C - \frac{1}{2}J(\text{C,H}) & \text{and} \\ M_C^{\text{H}\beta} &= \nu_C + \frac{1}{2}J(\text{C,H}) \end{aligned} \quad \text{Eq. 6.31}$$

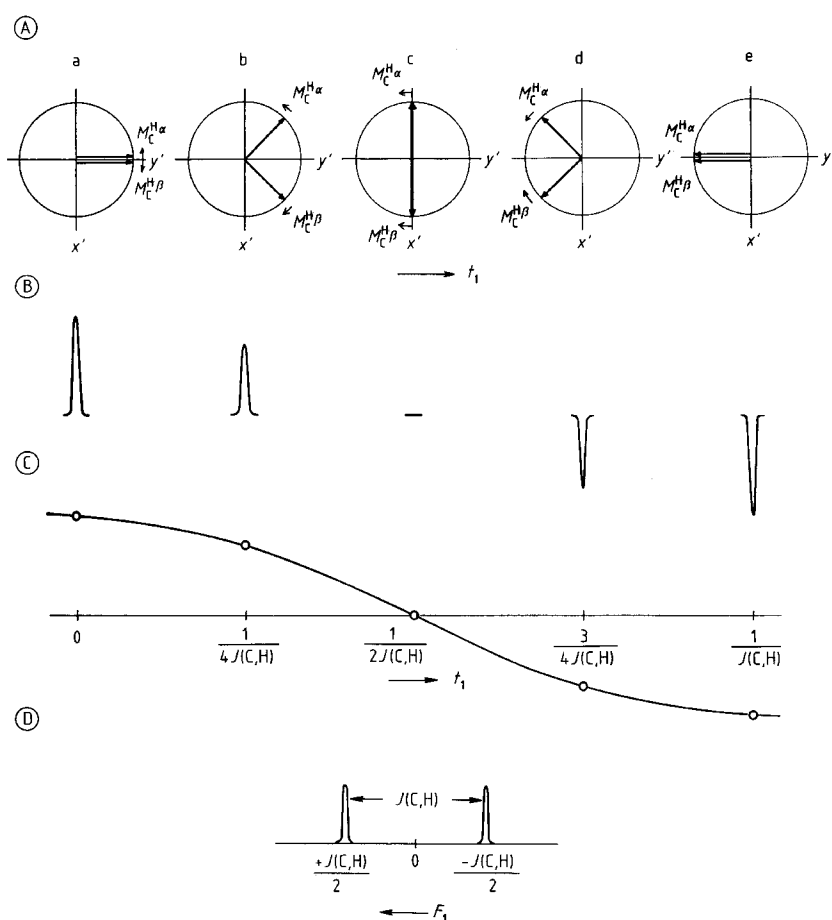
Assumed that the coordinate system, x',y' , rotates with the average of the Larmor frequency, ν_C , one vector rotates faster, the other slower, each by $J(\text{C,H}) / 2$. The rotations of the two vectors are indicated by small arrows in Figure 6.41. During the time t_1 , the two vectors of magnetization, $M_C^{\text{H}\alpha}$ and $M_C^{\text{H}\beta}$, will travel the angles ϕ_α and ϕ_β according to

$$\begin{aligned} \phi_\alpha &= 2\pi \left(\nu_C - \frac{1}{2}J(\text{C,H}) \right) t_1 & \text{and} \\ \phi_\beta &= 2\pi \left(\nu_C + \frac{1}{2}J(\text{C,H}) \right) t_1 \end{aligned} \quad \text{Eq. 6.32}$$

The phase difference between the two vectors can now be calculated according to

$$\Theta = \phi_\beta - \phi_\alpha = 2\pi \cdot J(\text{C,H}) \cdot t_1 \quad \text{Eq. 6.33}$$

After $t_1=[4 J(\text{C,H})]^{-1}$ (which is in the ms region), the phase difference between the two vectors is 90° , after $t_1=[2 J(\text{C,H})]^{-1}$ the difference is 180° , and after $t_1=[J(\text{C,H})]^{-1}$ the two vectors are in phase again, but their vectors now point into the $-y'$ direction. How do the



A Vector diagrams for the ^{13}C magnetization vectors of a AX two spin-system ($A=^1\text{H}$ and $X=^{13}\text{C}$) at different times t_1 . The coordinate system x', y', z rotates around z with the Larmor frequency ν_C . (**a** $t_1=0$, **b** $t_1=[4J(C,H)]^{-1}$, **c** $t_1=[2J(C,H)]^{-1}$, **d** $t_1=3[4J(C,H)]^{-1}$, **e** $t_1=[J(C,H)]^{-1}$).

B The ^{13}C NMR spectrum consists of singlets after a Fourier transformation with respect to t_2 (with ^1H BB decoupling). The amplitudes of the singlets depend on t_1 .

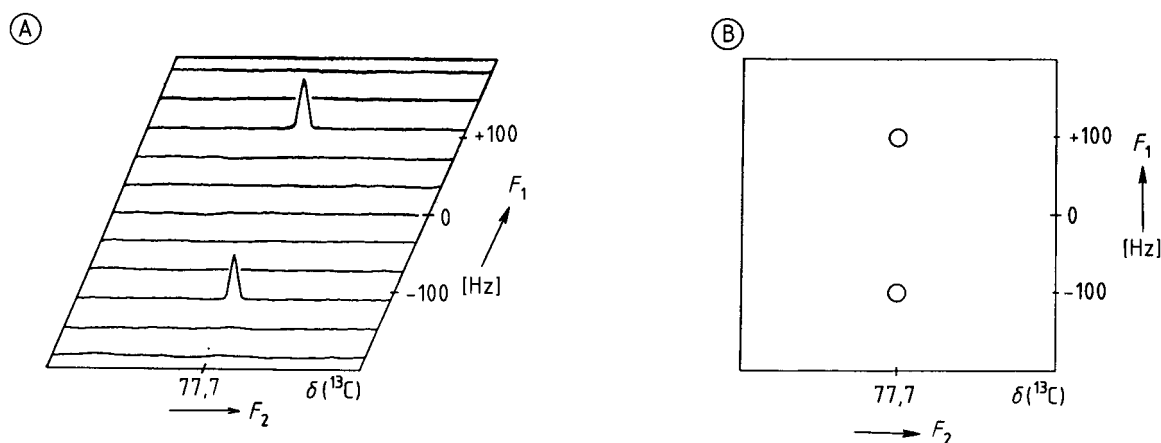
C The amplitude is modulated by $J(C,H)$

D By Fourier transform with respect to t_1 , two signals are obtained with a distance of $J(C,H)$ on the frequency axis F_1 .

Figure 6.41 Vector diagrams for the magnetization vectors of ^{13}C in an AX two spin system.

frequency spectra look like? Since the BB decoupler is turned on during the detection of the FID, all C, H-coupling is eliminated during this phase, and the F_2 spectra consist of singlets only. However the interaction between the nuclei during the time t_1 remains unaffected. To understand the t_1 effect, we consider the state of the system after a time $t_1 = [4J(C,H)]^{-1}$, when the phase difference is 90° . Switching on the BB decoupler eliminates the source for the different precession frequencies of $M_C^{H\alpha}$ and $M_C^{H\beta}$. Now, both rotate equally fast with an average Larmor frequency ν_C . However, their phase difference of 90° remains the same. The detector detects a signal with an amplitude that is proportional to the sum of the two vectors,

$M_C^{H\alpha}$ and $M_C^{H\beta}$. At $t_1=0$, both vectors are parallel, the sum of the vectors is largest and the amplitude of the signal has a maximum. At $t_1 = [2J(C,H)]^{-1}$ the component in y' direction is 0 and a signal cannot be detected. At $t_1 = [J(C,H)]^{-1}$, the signal is as large as at $t_1 = 0$, except for a loss due to relaxation, but it will have a negative amplitude. The signals that are obtained at different times t_1 are shown in Figure 6.41 B. The amplitude of the signal as a function of time is shown in Figure 6.41 C. A second Fourier transform with respect to time t_1 will thus yield two frequencies that differ by the coupling constant J . The F_2 spectrum thus contains the chemical shifts δ and the F_1 spectrum the coupling constants $J(C,H)$. In case of chloroform, all F_2 spectra consist of a singlet, whereas the F_1 spectrum consists of a doublet with a distance equal to the coupling constant (Figure 6.42).

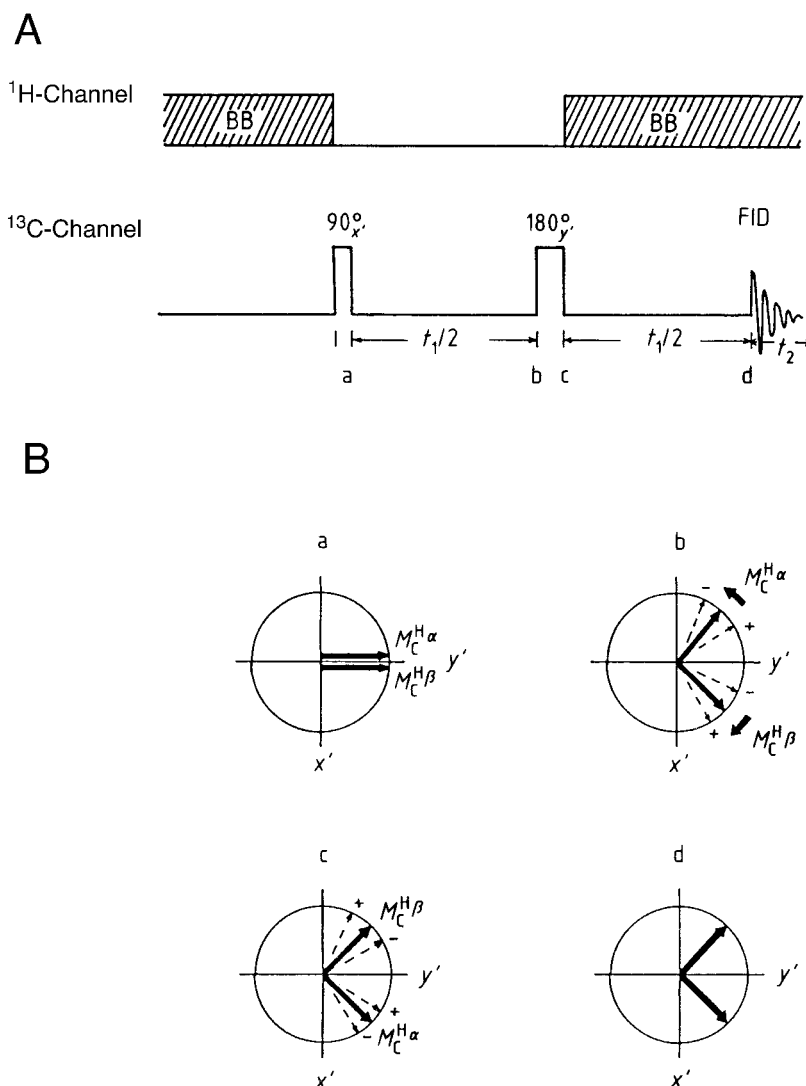


Schematic representation of a 2-D spectrum. **A** stacked plot. The F_2 -axis corresponds to the normal frequency axis with the δ -scale of the 1-D NMR spectrum. F_2 spectra are stacked for different F_1 values. **B** Contour plot. The contour plot is a slice through the stacked plot at a defined signal height. (Note that in the stacked plot, spectra are offset in F_1 direction to improve readability.)

Figure 6.42 Schematic representation of a 2-D spectrum.

6.10.1.1 Two-dimensional J-resolved NMR spectroscopy.

In the experiment that was discussed in the last section, two Fourier transformations were performed that gave rise to a two dimensional spectrum. In such a spectrum, the chemical shifts were given in one dimension (F_2 -axis) and the C,H-coupling constants were given in the second dimension (F_1 -axis). Since the coupling took place between hetero nuclei, we discussed the simplest case of a heteronuclear 2-dimensional J-resolved NMR spectroscopy (J, δ -spectroscopy). There are different variants of this kind of 2-D spectroscopy, from which the so-called gated decoupling method shall be discussed. The pulse sequence of this method is given in Figure 6.43 To understand the effect of the pulse sequence, we shall again

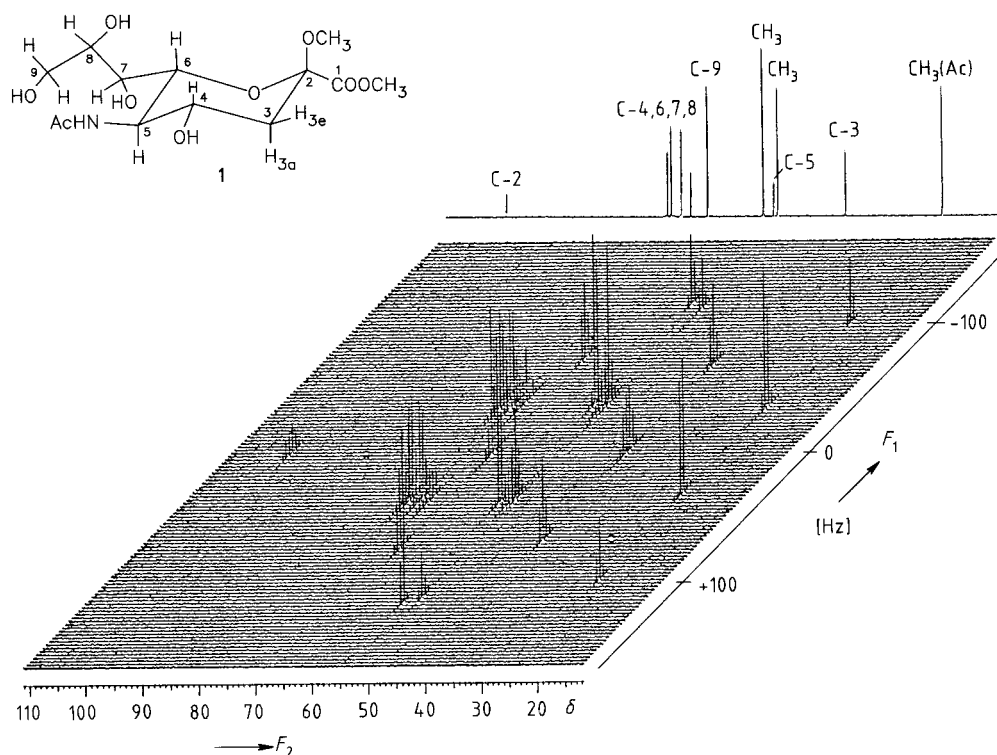


Heteronuclear two-dimensional J -resolved ^{13}C -NMR spectroscopy. **A** Pulse sequence. **B** Evolution of the transversal ^{13}C magnetization vectors M_C for an AX-two-spin system, with $A=^1\text{H}$ and $X=^{13}\text{C}$ in a rotating coordinate system (x', y' -plane). $M_C^{H\alpha}$ and $M_C^{H\beta}$ correspond to the ^{13}C magnetization vectors for the molecules with a proton in the α or in the β state, respectively. Diagrams in **B** are drawn corresponding to the times a, b, c, and d indicated in **A**. The spin-system is developing with the coupling constant $J(\text{C}, \text{H})$ and is partially dispersed because of field inhomogeneities (slower and faster than average precession of the spins is indicated by + and -). In practice, the broadband decoupler is switched on in the first half of the evolution phase ($t_1/2$) and during detection.

Figure 6.43 Heteronuclear 2-D J -resolved ^{13}C -NMR spectroscopy . Pulse Sequence and development of the spin-system.

consider the AX two spin system of chloroform ($^{13}\text{CHCl}_3$). The basis for this experiment is the pulse sequence $90^\circ_{x'}-t-180^\circ_{y'}-t$ (echo). Compare to the pulse sequence given in Figure 6.40 this sequence now has a $180^\circ_{y'}$ pulse in y -direction after half of the evolution time $t_1/2$. In addition, the new sequence also has the broad band decoupler turn on during the second half of the evolution time. To illustrate the behavior of the spin system, vector diagrams are drawn in Figure 6.43B. The coordinate system x' , y' , and z again rotates with

the mean frequency ν_C . The first pulse $90^\circ x'$ in x' -direction rotates the two magnetization vectors $M_C^{H\alpha}$ and $M_C^{H\beta}$ to the y' -axis. During $t_1/2$ ms the two vectors will separate and $M_C^{H\beta}$ is the vector that rotates faster and $M_C^{H\alpha}$ the vector that rotates slower. In the diagram (Figure 6.43 B), the arrows indicate the relative directions of motion. Due to field inhomogeneities, the motions will be partially disperse either faster or slower than average. A $180^\circ_{y'}$ pulse in y' -direction will mirror the spins with respect to the y' axis (Figure 6.43 C). After an additional time of $t_1/2$ ms, the spins would be refocused in the y' -axis, if the 1H broad-band decoupler were turned off. However, if the broad band decoupler is turned on during the second $t_1/2$ ms, the vectors $M_C^{H\alpha}$ and $M_C^{H\beta}$ will now rotate equally fast (with ν_C , which is the speed of the coordinate system), but their phase difference will remain the same.

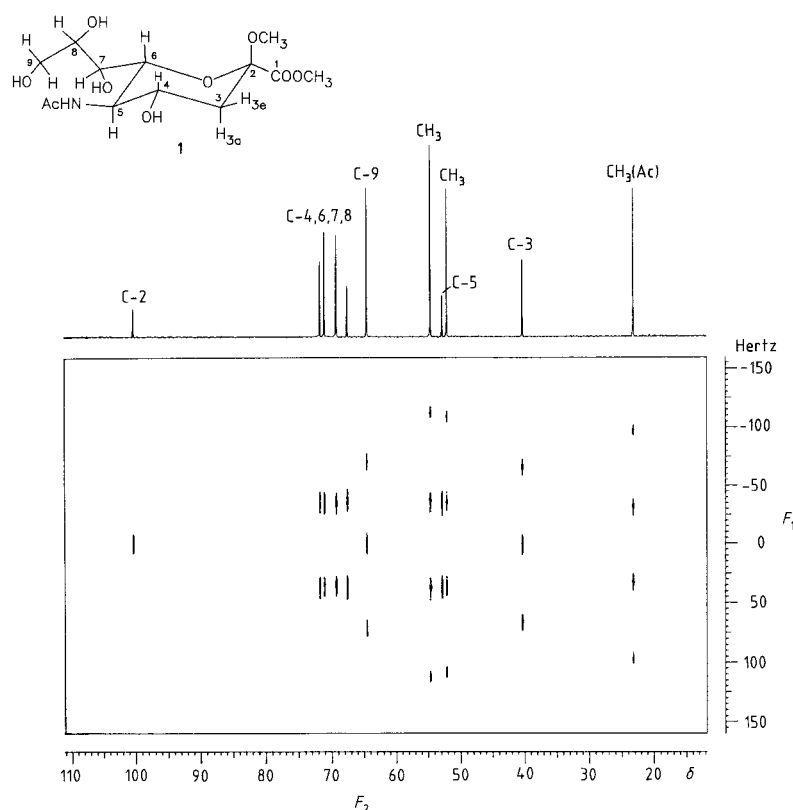


Stacked plot of the heteronuclear J -coupled 100.6 MHz ^{13}C NMR spectrum. On the upper edge, the projection of the multiplets is shown. This spectrum corresponds to the 1H BB-decoupled ^{13}C NMR spectrum. Multiplets parallel to the F_1 -axis indicate how many H-atoms are bound to the corresponding carbon atom. The distance between the signals in the multiplets corresponds to $J(C,H)/2$, since the spin-system developed for $0.5 t_1$ ms. Signals of the quarternary C-atoms of the carboxy and of the acetamide-group are not shown.

Figure 6.44 Heteronuclear J -coupled 2D-NMR spectrum of neuraminic acid.

The partial dispersion of the spins that are caused by field inhomogeneities, however, is

equalized after $t_1/2$ ms by the $180^\circ_{y'}$ -pulse. After a time t_1 , the signal may be recorded. This signal is proportional to the sum of the two vectors, $M_C^{H\alpha}$ and $M_C^{H\beta}$. This sum depends on the phase difference, which is again dependent on the coupling constant $J(C,H)$. The ^{13}C -NMR signal is thus modulated by $J(C,H)$. The Fourier transformation with respect to t_2 results in a singlet that is modulated by $J(C,H)$. The Fourier transformation with respect to t_1 results in a doublet on the F_1 -axis that has a distance of $1/2 J(C,H)$ (the system was allowed to develop only $t_1/2$).

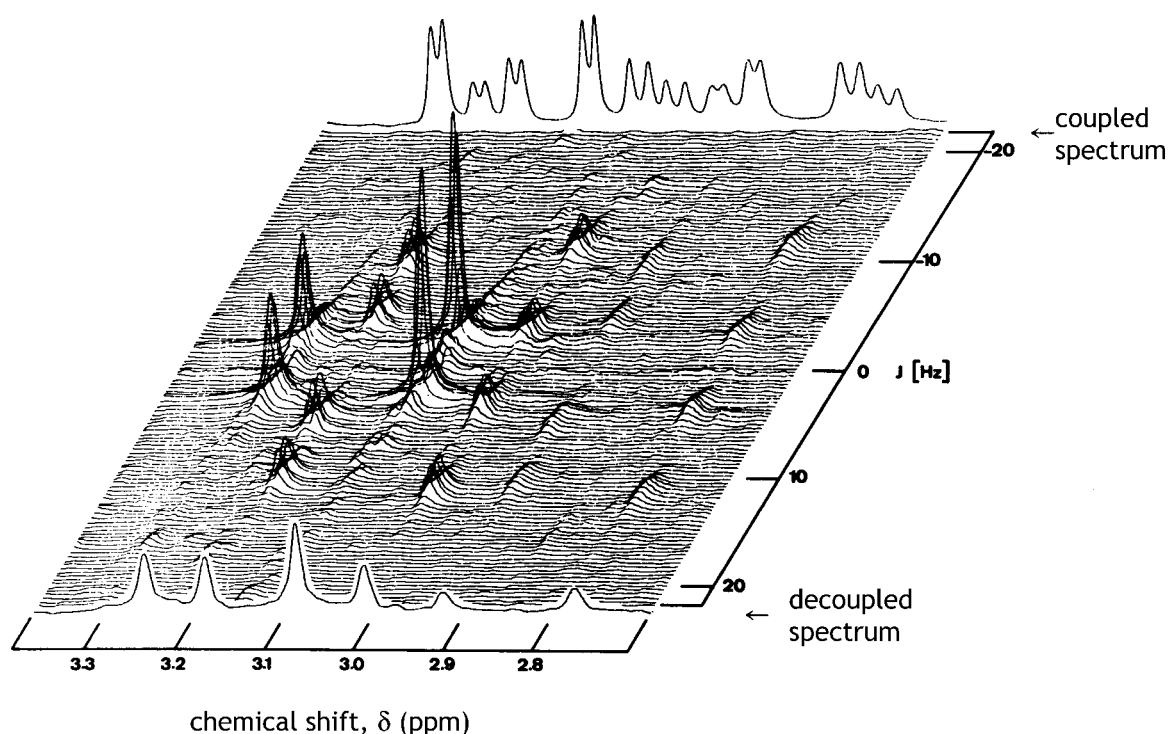


Contour plot

Figure 6.45 Contour plot of the spectrum shown in Figure 6.44

For other molecules, the F_2 spectrum will consist of as many singlets as there are different ^{13}C -atoms. On the t_1 -scale, all signals are modulated with the corresponding C,H-coupling constants. Parallel to the F_1 axis, the multiplets are obtained, that result from the coupling with the protons: singlets for quaternary carbons, doublets for C-H (tertiary carbons), triplets for CH_2 (secondary carbons) and quadruplets for CH_3 -groups. A 100.6 MHz 2-D ^{13}C -NMR spectrum recorded by this method is shown in Figure 6.44 for a neuraminic acid derivative. Parallel to the F_2 -axis, the projection of the 2-D NMR spectrum represents the proton decoupled

^{13}C -NMR spectrum (shown at the upper edge of the 2D spectrum). Parallel to the F_1 axis, the multiplet structure of each signal is obtained. The multiplet structure makes it possible to decide which of the signals belongs to a CH_3 , CH_2 , CH , or to a quaternary carbon. The assignment of the signals is shown as it can be derived from the chemical shifts and the multiplet structure of the signal. From the separation of 2 lines in the multiplets, one can estimate half the value of the coupling constant $^1J(\text{C,H})$. The contour diagram of the same spectrum is shown in Figure 6.45.



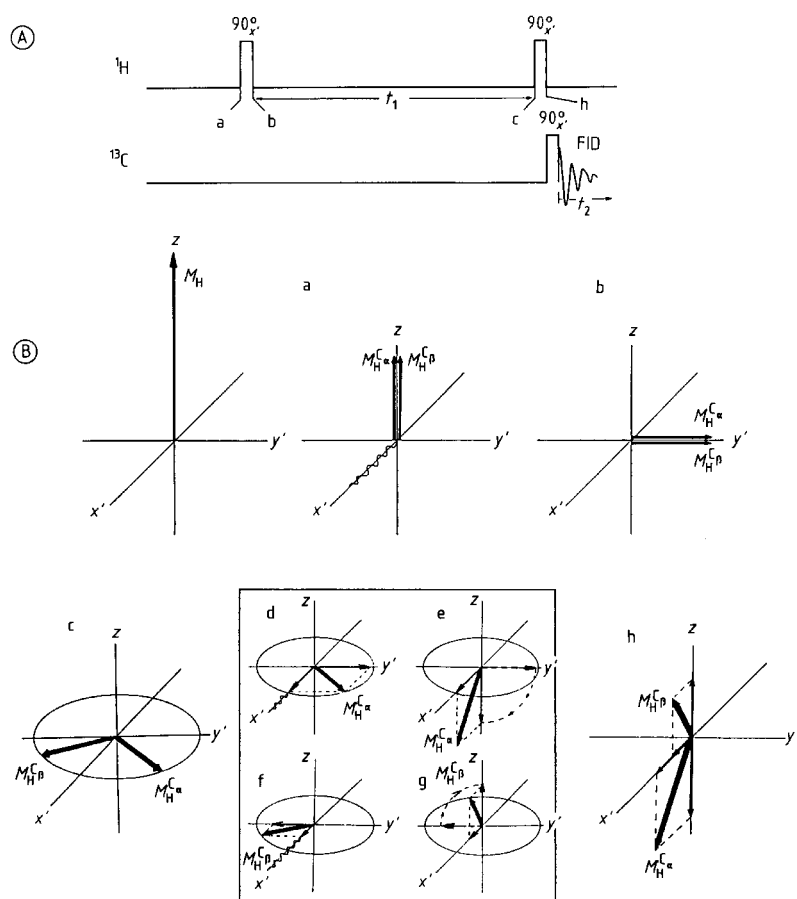
Part of the two-dimensional 360 MHz ^1H -NMR spectrum of a solution of the amino acids alanine, isoleucine, threonine, histidine and tryptophan. The non-decoupled spectrum is shown at the upper edge, the decoupled spectrum at the lower edge. The decoupled spectrum is the projection of the 2D spectrum along the J -axis.

Figure 6.46 Two dimensional J-coupled ^1H -NMR spectrum of a solution of different aromatic amino acids.

6.10.2 2-dimensional correlated spectroscopy.

6.10.2.1 Two-dimensional heteronuclear (H, C)-correlated NMR spectroscopy (H,C-COSY).

Many problems of signal assignment can be solved elegantly using the two dimensional chemical shift correlated spectroscopy (COSY). For a simple example, we again consider the two spin system of chloroform ($^{13}\text{CHCl}_3$) and perform an NMR experiment using the pulse sequence depicted in Figure 6.47 is used to obtain the spectrum. How the pulse sequence,



2-dimensional H, C-correlated spectroscopy. **A** pulse sequence. **B** vector diagrams that illustrate, how the ^1H magnetization vectors $M_H^{C\alpha}$ and $M_H^{C\beta}$ of an AX-two spin system ($A=^1\text{H}$ and $X=^{13}\text{C}$) develop in a rotating coordinate system. The vector diagrams a to c correspond to the times given in A. Diagrams d, e, f, and g depict the effect of the second 90°_x pulse separately for $M_H^{C\alpha}$ and $M_H^{C\beta}$ in the ^1H channel. The diagram h corresponds to the state in the ^{13}C channel before the 90°_x detection pulse.

Figure 6.47 2-dimensional H,C correlated NMR spectroscopy. A Pulse sequence. B Vector diagrams

$90^\circ_{x'} \rightarrow t_1 \rightarrow 90^\circ_{x'}$, which is applied to the ^1H -channel, affects the macroscopic magnetization of the ^1H vectors, M_H^{Ca} and M_H^{Cb} , is shown in Figure 6.47 B. M_H^{Ca} and M_H^{Cb} correspond to the magnetizations of the chloroform molecules with ^{13}C in the α and in the β state. The first $90^\circ_{x'}$ -pulse rotates both magnetization vectors from the z-axis to the y' -axis. In the following evolution phase, t_1 , both vectors rotate with the Larmor frequencies

$$\nu_H - \frac{1}{2}J(\text{C},\text{H}) \quad \text{and} \quad \nu_H + \frac{1}{2}J(\text{C},\text{H}) \quad \text{Eq. 6.34}$$

ν_H is the Larmor frequency without coupling, i. e. the resonance of the protons in $^{12}\text{CHCl}_3$. In the time t_1 , M_H^{Ca} passes over the angle φ_α , M_H^{Cb} over the angle φ_β :

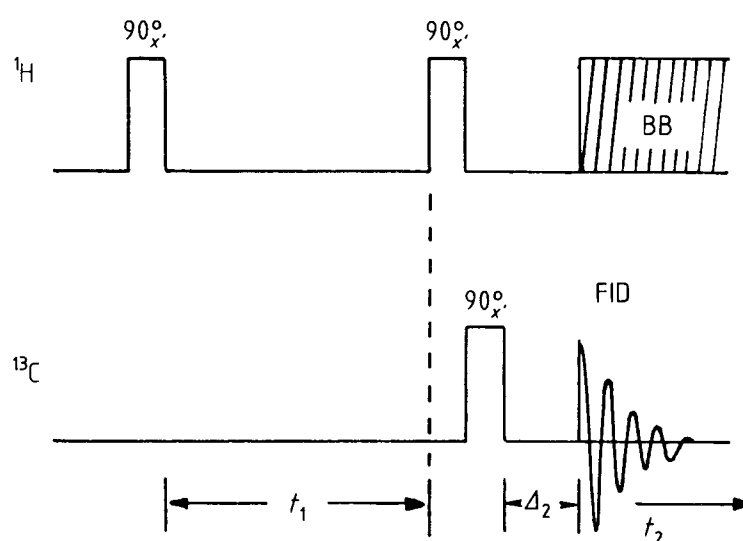
$$\begin{aligned} \varphi_\alpha &= 2\pi \left(\nu_H - \frac{1}{2}J(\text{C},\text{H}) \right) t_1 \quad \text{and} \\ \varphi_\beta &= 2\pi \left(\nu_H + \frac{1}{2}J(\text{C},\text{H}) \right) t_1 \end{aligned} \quad \text{Eq. 6.35}$$

The phase difference Θ is dependent only on the time t_1 and the coupling constant $J(\text{C},\text{H})$:

$$\Theta = \varphi_\beta - \varphi_\alpha = 2\pi J(\text{C},\text{H}) t_1 \quad \text{Eq. 6.36}$$

For $t_1 = [4 J(\text{C},\text{H})]^{-1}$: $\Theta = 90^\circ$ and for $t_2 = [2 J(\text{C},\text{H})]^{-1}$: $\Theta = 90^\circ$. In Figure 6.47 C, the status of the magnetization vectors is shown after an arbitrary time t_1 (in the range of milliseconds). Since the frequencies of the 2 vectors do not coincide with the Larmor frequency of the rotating coordinate system, the vectors M_H^{Ca} and M_H^{Cb} are at an angle towards the y' axis. The vector M_H^{Ca} has components in the y' and in the x' axis. The second $90^\circ_{x'}$ -pulse in the ^1H channel rotates the y' -component to the $-z$ direction, while the x' remains unaffected. The new direction of the total magnetization M_H^{Ca} is determined by the components in x' -direction and in $-z$ direction. In this example, the vector points to the lower front quadrant and is in the x', z -plane. The same observation is made for the y' -component of M_H^{Cb} : this component, however is transformed to the $+z$ -direction, and M_H^{Cb} is found in the upper front quadrant in the x', z -plane. For further considerations, only the z-components of M_H^{Ca} and M_H^{Cb} are necessary. These longitudinal magnetizations are proportional to the populations differences between energy levels 1 and 3 (M_H^{Ca}) and between energy levels 2 and 4 (M_H^{Cb}). Two conclusions may be drawn: 1. By the pulse sequence $90^\circ_{x'} \rightarrow t_1 \rightarrow 90^\circ_{x'}$ the population ratios have changed compared to the initial state. The change in the population ratio is determined by t_1 and may even lead to a stronger population of level 3. 2. The state of the spin system depends on t_1 and on the angles φ_α and φ_β . These angles are dependent on the Larmor frequency ν_H and on the coupling constant $J(\text{C}, \text{H})$.

How does the magnetization of the protons M_H that is induced by the pulse sequence in the ^1H -channel affect the ^{13}C -NMR spectrum? The amplitude of the ^{13}C -NMR signal is determined by the population ratio after the second 90° pulse (compare the SPI experiment, section 6.9.3). The ^{13}C -NMR signal is not enhanced by a fixed factor, but is modulated as a function of t_1 by the Larmor frequencies of the protons. The magnetization vectors M_H^{Ca} and M_H^{Cb} — and therefore the signal intensities of the ^{13}C -NMR peaks — are affected to the same extent, but with opposite signs. The 90°_x detection pulse in the ^{13}C channel rotates the longitudinal vectors to the $+y'$ and $-y'$ -axes. During the detection phase t_2 , both vectors precess with the corresponding transition frequencies X_2 and X_1 and induce the interferogram in the detector.



Extended pulse sequence to simplify the 2-dimensional H,C-correlated NMR-spectrum. After the 90°_x -pulse in the ^{13}C channel, a delay time Δ_2 is introduced, before the broad band decoupler is turned on and the FID is recorded.

Figure 6.48 Extended pulse sequence to simplify the H, C-COSY spectrum.

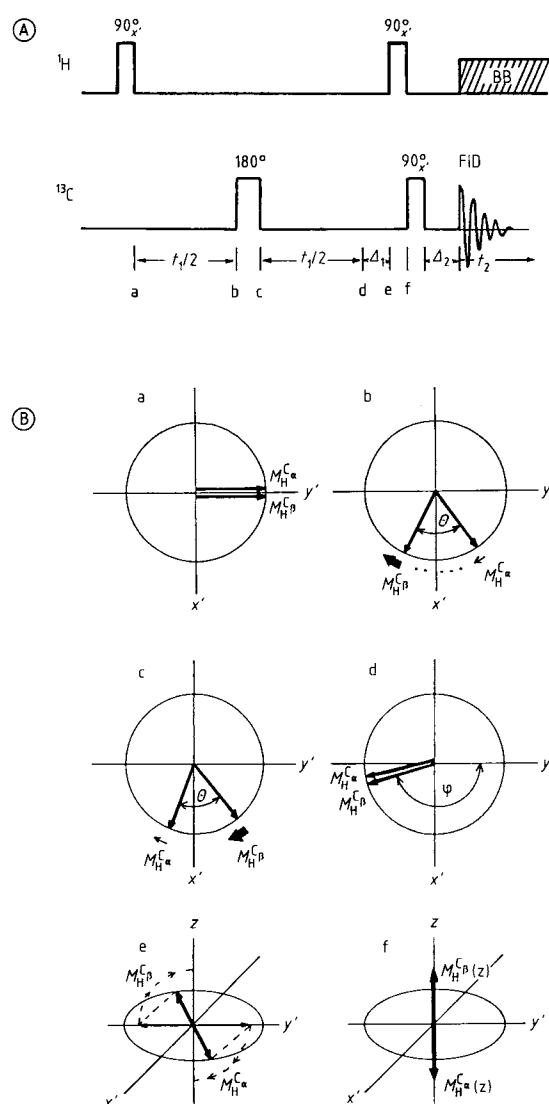
Fourier Transformation (FT) with respect to t_2 results in two signals on the F_2 axis, which are modulated by t_1 and by the resonance frequencies of the protons. If n spectra are recorded with different t_1 times and a FT is performed with respect to t_1 , then a two-dimensional spectrum with 4 signals is obtained, two of which have a positive, the two others a negative amplitude. The F_1 -axis describes the ^1H resonances and the F_2 -axis the ^{13}C resonances. A and X are the frequencies of the corresponding transitions of ^1H and ^{13}C (which are usually given as δ values). The spectrum parallel to the F_2 -axis corresponds to the coupled ^{13}C -NMR spectrum, the spectrum parallel to the F_1 -axis corresponds to the coupled ^1H -NMR-spectrum. In a simple spectrum, such as that of chloroform with only two coupled nuclei, spectra are still easy to interpret. For larger molecules, this experiment must be changed to reduce

complexity of the spectrum.

Unfortunately, one cannot simply turn on the broad band decoupler to reduce the carbon doublets to singlets, because the signals of interest would be destroyed (M_C^{Ha} and M_C^{Hb} have opposite signs, thus add up to zero). Only without BB-decoupling, two signals are found. A different state is observed, if we insert a delay time $\Delta_2 = [2 J(C, H)]^{-1}$ between the $90^\circ_{x'}$ -detection pulse and the detection of the FID (Figure 6.48). During this time Δ_2 the faster magnetization vector M_C^{Hb} has progressed 180° more than M_C^{Ha} , and both vectors are again in phase, despite their differences in precession frequency. If the BB-decoupler is switched on in this moment, both vectors will precess equally fast. After FT of the FID with respect to t_2 , only one signal is observed at ν_C . In a modified experiment, n spectra are obtained for different values of t_1 , that are chosen to be equidistant with a difference of a few ms. The n ^{13}C NMR spectra are modulated by the proton resonance frequencies, according to the polarization transfer in the ^1H channel. After a second FT with respect to t_1 , the 2-dimensional spectrum is obtained that consist now of 2 signals that correspond to one transition of ^{13}C and two transitions of ^1H .

Finally, we can use the pulse sequence depicted in Figure 6.49 to reduce the two resonance lines to one single signal. New is a 180° pulse in the ^{13}C channel after exactly $t_1/2$ ms and a delay time Δ_1 before the second pulse in the ^1H channel. The vector diagram in Figure 6.49 B, illustrates the experiment. The first 90° pulse rotates the two ^1H magnetization vectors M_H^{Ca} and M_H^{Cb} to the y' -axis. According to their Larmor frequencies, $\nu_H - J(C, H)/2$ and $\nu_H + J(C, H)/2$ there is a different precession of the two vectors. After time $t_1/2$, the phase difference between the vectors is $\Theta = \pi J(C, H) t_1$. By a 180° pulse in the ^{13}C -channel α - ^{13}C nuclei become β - ^{13}C nuclei and vice versa. This means, M_H^{Ca} becomes M_H^{Cb} and M_H^{Cb} becomes M_H^{Ca} . Now the faster vector is behind the slower vector in the vector diagram (thick and thin arrow). After another time $t_1/2$ ms the faster vector has reached the slower vector and both are in phase. The total angle ϕ that the vectors have passed is only dependent on the Larmor frequency of the protons (without coupling with the ^{13}C nuclei). A pulse that would directly follow after time t_1 would not result in a polarization of the ^{13}C NMR signal. However if a delay time of Δ_1 is introduced before the second pulse $90^\circ_{x'}$ in the ^1H -channel, the vectors M_H^{Cb} and M_H^{Ca} have progressed again by different angles that have a difference of 180° after $\Delta_1 = [2 J(C, H)]^{-1}$ ms. A $90^\circ_{x'}$ pulse in the ^1H channel causes the y' -components of the vectors to rotate to the $-z$ and $+z$ directions, and therefore the induction of polarization. How large this polarization is, depends on the angle ϕ . If both vectors are positioned along the y' -axis, the polarization has a maximum. If they are positioned along the x' -axis, the polarization is zero. The angle ϕ , by which the vectors have progressed, is a function of the Larmor frequency ν_H of the decoupled protons. The evolution of the spin system continues during the time Δ_1 , but this time is a constant among all spectra that are collected with different times t_1 . The state of polarization that affects the intensities of the ^{13}C resonances, is therefore exclusively determined by the Larmor frequency ν_H of the protons. The subsequent events in the experiment

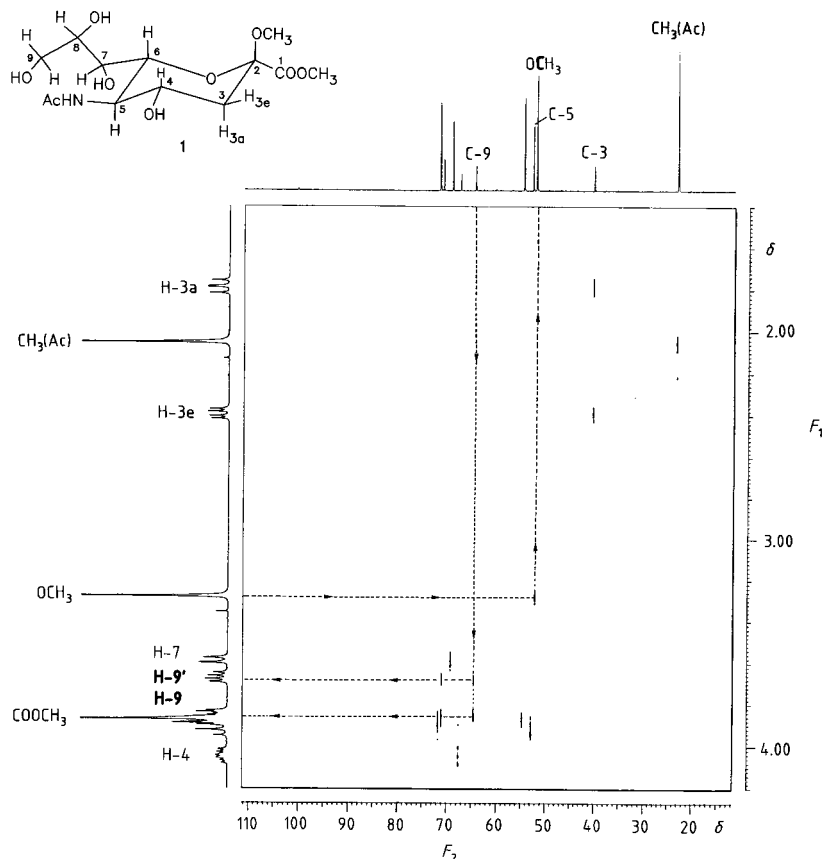
are a 90° pulse in the ^{13}C channel, which rotates the magnetization vectors M_{C}^{Ha} and M_{C}^{Hb} in the $+y'$ and the $-y'$ directions. After a constant time $\Delta_2 = [2 J(\text{C}, \text{H})]^{-1}$ ms these vectors are again in phase. In this moment, the BB-decoupler is turned and it will stop the coupling between ^{13}C and ^1H . The first Fourier transform with respect to t_2 results in a signal at ν_{C} . If spectra were recorded with different times t_1 and with $\Delta_1 = \Delta_2 = [2 J(\text{C}, \text{H})]^{-1}$ ms then the signal intensities are modulated with ν_{H} . A second Fourier transform with respect to t_1 results in a two-dimensional spectrum (F_1, F_2) which consists of only one signal with the coordinates (ν_1, ν_2) .



A. Pulse Sequence for a 2-dimensional H,C-correlated NMR experiment in which the 2D spectrum of a AX Twospin system is reduced to one signal.
 B. Vector diagrams illustrate the positions of the ^1H -magnetization vectors $M_{\text{H}}^{\text{C}\alpha}$ and $M_{\text{H}}^{\text{C}\beta}$ and their z-components at the times given in A. In diagrams a through d only the x-y plane is depicted.

Figure 6.49 Pulse sequence for the 2-dimensional H, C -correlated experiment.

The signal is most intense for $\Delta_1 = [2 J(\text{C}, \text{H})]^{-1}$ ms. Furthermore, signal intensity is amplified by transfer of magnetization from the more sensitive ^1H nucleus to the less sensitive ^{13}C nucleus. The time period between the end of t_1 and the beginning of t_2 is called “mixing”. The same experiment can be performed for multi-spin systems.



2-dimensional H,C correlated 100.6 MHz-NMR spectrum of a neuraminic acid derivative. On the left, the 1-dimensional ^1H -NMR spectrum is shown, and above, the projection of the 2-dimensional NMR spectrum to the F_2 -axis, the ^{13}C -NMR spectrum, is depicted.

Figure 6.50 2-dimensional H,C -correlated 100.6 MHz NMR spectrum of a neuraminic acid derivative. The spectrum was recorded in D_2O .

Figure 6.50 shows the 2-dimensional H,C correlated 100.6 MHz NMR spectrum of a neuraminic acid derivative. On the upper edge, the 1-dimensional (1-D) ^{13}C -NMR spectrum is shown. This spectrum is obtained by projecting the 2-D peaks to the F_2 -axis. Those signals can be recognized that are directly connected to ^1H -atoms. The three quarternary carbons do not appear as correlation peaks. On the left hand side, the 1-dimensional ^1H -NMR spectrum is shown. By characteristic chemical shifts and multiplicities, a few assignments are clearly defined. In the ^1H -NMR spectrum, these are the assignments of the three methyl signals and also those of H-3a, H-3e, and possibly H-4 and H-7. In the 1-D ^{13}C -NMR spectrum, the

resonances of the methyl carbon of the NAc-group and C-3, C-5, C-9 are known. Known are also the chemical shifts of the two O-CH₃ signals, but they cannot be assigned unambiguously. For analysis we start with the assigned signals of the ¹H resonances. The correlation peaks in the 2-D spectrum now give us the chemical shifts of connected carbon atoms in the 1-D ¹³C NMR spectrum. In addition to the already known resonances, we can now assign the chemical shifts of the two methoxy groups as well as those of C-4 and C-7.

If we start the analysis with the assigned ¹³C resonances, we are now able to assign the chemical shifts of H-5, H-9, and H-9', in addition to those ¹H-nuclei, that were assigned on the basis of the ¹H-NMR spectrum alone. For C-3 and C-9 we obtain two correlation peaks, because these C-atoms are bound to two diastereotopic H-atoms.

With the new assignments, the NMR spectrum, the 1-dimensional ¹H and the ¹³C NMR spectra are almost completely analyzed. Only the assignments of H-6 and H-8, and C-6 and C-8 are missing. This assignment cannot be made on the basis of the (H,C)-correlated 2-D NMR spectrum, since resonances are too close in both the ¹³C and the ¹H-NMR spectrum. The results are summarized in Table 5.

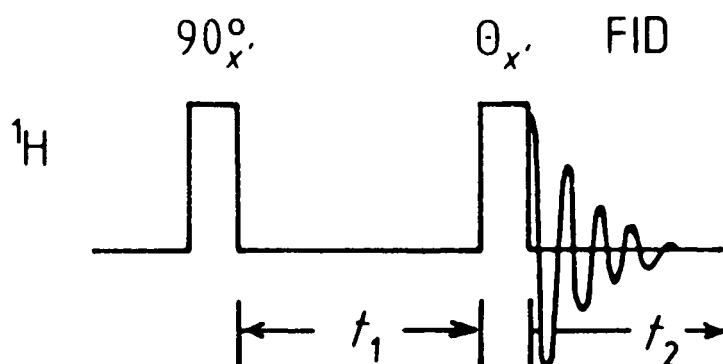
basis for assignment	assignment
H-4	C-4
H-7	C-7
OCH ₃ (ketosid)	OCH ₃
OCH ₃ (ester)	OCH ₃
C-5	H-5
C-9	H-9
C-9	H-9'

Table 5 Results of the analysis of the (H, C) COSY 2D-NMR spectrum

The COSY method is widely used in the analysis of large molecules, such as those found in biochemistry and natural substances. A major advantage is that the relatively large chemical shifts of the ¹³C nuclei can be combined with those of the ¹H nuclei.

6.10.2.2 Two-dimensional homonuclear (H, H)-correlated NMR spectroscopy (H,H-COSY).

The 2-dimensional homonuclear (H,H)-correlated NMR experiment results in spectra that contain ^1H chemical shifts on both frequency axis. This method became known as correlated spectroscopy (COSY). It is based on the pulse sequence $90^\circ_{x'} - t_1 - \Theta_{x'}$ (Figure 6.51).



Pulse-Sequence for a 2-dimensional homonuclear (H,H)-correlated NMR experiment (COSY). The variable is t_1 . $\Theta = 90^\circ_{x'}$ or $45^\circ_{x'}$, sometimes $\Theta = 60^\circ_{x'}$.

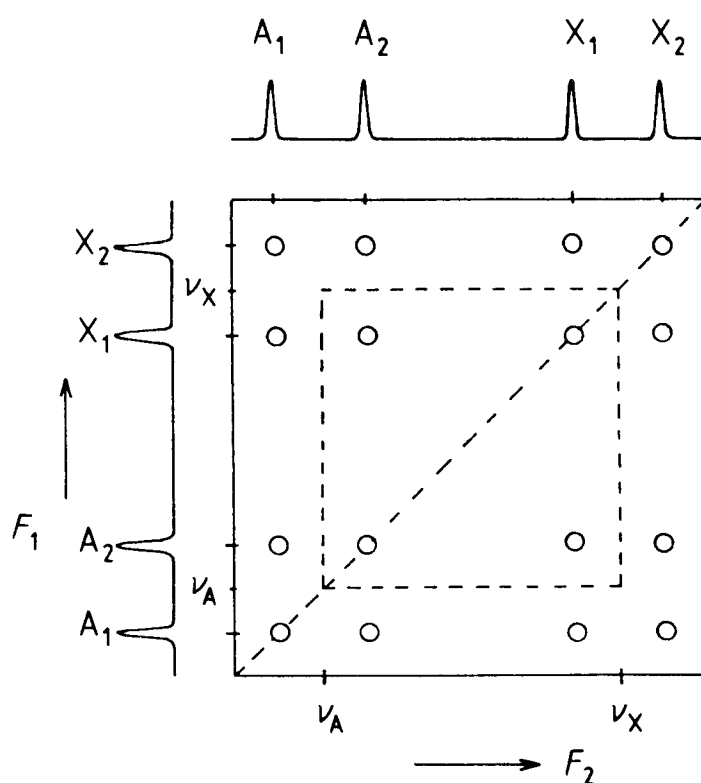
Figure 6.51 Pulse sequence for the 2-dimensional homonuclear (H,H)-correlated NMR experiment (COSY).

We shall consider the experiment for an AX two spin system with a second pulse of $\Theta_{x'} = 90^\circ$. A and X are ^1H nuclei with a coupling constant of $J(\text{A},\text{X})$. The pulse sequence therefore is $90^\circ_{x'} - t_1 - 90^\circ_{x'}$. In contrast to the heteronuclear C,H-COSY experiment, there is an important difference: the first pulse $90^\circ_{x'}$ affects the magnetization vectors of both the A and X nuclei, M_A and M_X , which are rotated to the y' -axis. Because of the coupling $J(\text{A},\text{X})$ there are two macroscopic magnetization vectors, $M_A^{x\alpha}$ and $M_A^{x\beta}$, depending on the state of the X nucleus, which may be an α or a β -state. Similarly, we must also consider two M_X vectors, $M_X^{A\alpha}$ and $M_X^{A\beta}$. These vectors rotate in the x, y -plane with the frequencies $\nu_A \pm J(\text{A},\text{X})/2$ and $\nu_X \pm J(\text{A},\text{X})/2$ around the z -axis. Within the time t_1 , which is the variable in the COSY experiment, the 4 magnetization vectors will separate within the x, y -plane because of their different frequencies. After a time t_1 , each of the four vectors has a component in x' and in y' direction. The following second $90^\circ_{x'}$ -pulse rotates the y' -component to the z axis, either in $-z$ or in $+z$ direction. This step includes a polarization transfer (compare section 6.9.3) To which extent magnetization is transferred, depends on the state of the spin system at time t_1 , and therefore on the Larmor frequencies ν_A, ν_X and on the coupling constant $J(\text{A},\text{X})$. The x' - components of the magnetization vectors, which are also dependent on the evolution of the spin state and which continue to rotate in the x', y' -plane, result in a free

induction decay, which after FT with respect to t_2 , yield a four line AX spectrum with the frequencies:

$$\begin{array}{llll} \nu_A + \frac{1}{2}J(A,X) & (A_1) & \nu_A - \frac{1}{2}J(A,X) & (A_2) \\ \nu_X + \frac{1}{2}J(A,X) & (X_1) & \nu_X - \frac{1}{2}J(A,X) & (X_2) \end{array}$$

These frequencies correspond to the transitions A_1 , A_2 , X_1 , X_2 that are depicted in Figure 6.39. The signals are modulated as a function of t_1 with these four frequencies. The second Fourier transform with respect to t_1 therefore leads to a two-dimensional spectrum with four groups that each contain 4 signals. Two of these groups are centered around ν_A , ν_A and ν_X , ν_X , the diagonal peaks. The other two are centered around ν_A , ν_X and ν_X , ν_A , the so called correlation peaks or cross peaks. Diagonal and crosspeaks form the corners of a square. *The important feature of the 2-D spectrum is that the correlation peaks (cross peaks) are always found when two nuclei are coupling by j-coupling.* Within each group the signals are separated

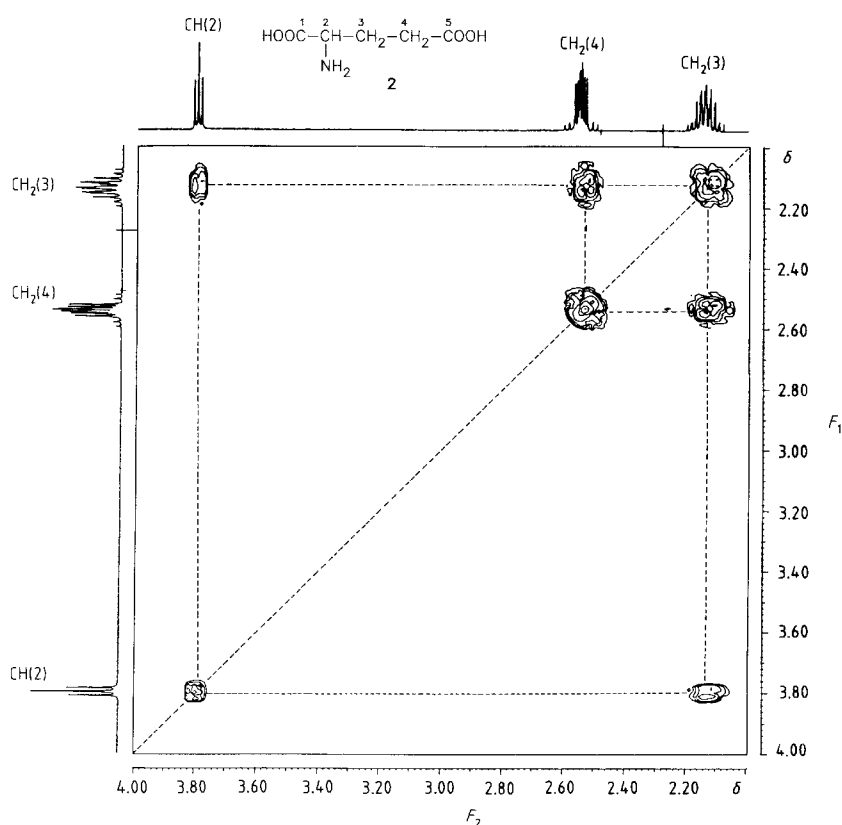


Scheme of a COSY spectrum of an AX two spin system with $A=X=H$. Shown are absolute values. In a real spectrum, signals on the diagonal are dispersion signals that may also have a negative amplitude. The diagonal peaks and the cross peaks of coupling nuclei form the corners of squares.

Figure 6.52 Scheme of a homonuclear COSY spectrum.

by the coupling constant $J(A,X)$ in each dimension (F_1 -axis and F_2 -axis). The projection of the COSY spectrum in each dimension F_1 or F_2 corresponds to the 1-dimensional ^1H -NMR spectrum (Figure 6.52). Figure 6.52 shows the 2D homonuclear COSY spectrum in form of a contour plot schematically with the absolute values of the signals.

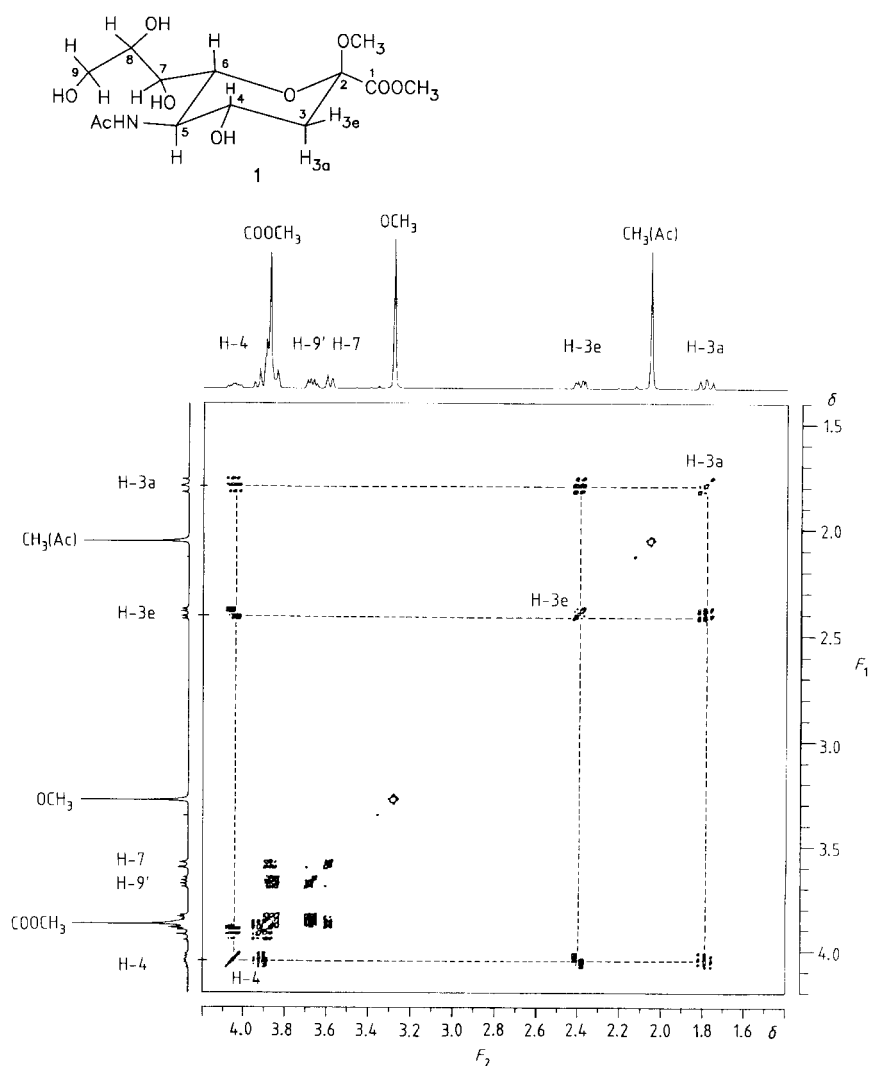
If a proton couples with more than one neighboring proton, then the diagonal peak is found in the corner of more than one square. In this way, the chemical shifts of coupled nuclei can be determined even in complex spectra. Therefore, the COSY experiment is an important tool in the assignment of ^1H resonances. It is superior to the 1D NMR experiment and various decoupling experiments, because the relations and chemical shifts between all coupling nuclei are obtained at once, with a single experiment.



500 MHz COSY-90 spectrum of glutamic acid. On the upper and on the left edge, the 500 MHz 1-dimensional ^1H -NMR spectra are shown. The dashed lines connect the diagonal and the correlation peaks, which indicate the protons that couple by scalar coupling. The diagonal peak of the C-3 protons is located in the corner of two squares, since these protons couple with the protons on C-3 and on C-4.

Figure 6.53 500 MHz COSY spectrum of glutamic acid.

Figure 6.53 shows the (H,H) COSY-90 spectrum of glutamic acid. On the left and on the upper edge, the 1-dimensional ^1H NMR spectra are shown. On the diagonal, three multiplets are observed, which correspond to the three multiplets of the one-dimensional spectrum. With these multiplets and the correlation peaks it is clear which of the protons are coupling. Since the protons on C-3 couple with the protons on C-4 and with the protons on C-2, the multiplet of the C-3 protons is found in the common corner of two squares. By a suitable choice of experimental conditions, even far reaching smaller coupling can be detected. A disadvantage of the method is, that in larger molecules the COSY contour diagram can easily be too crowded. If there are only minor differences in the chemical shifts of coupling nuclei,



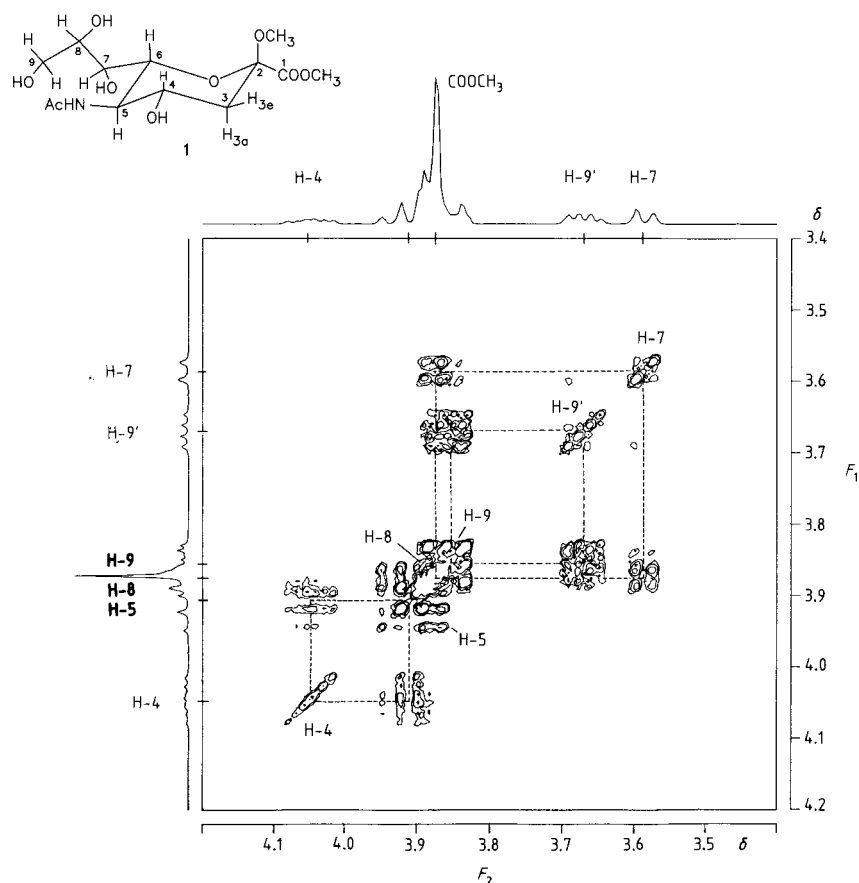
400 MHz-COSY-45 spectrum of a neuraminic acid derivative. Above the 2D-spectrum, the projection of the 2D-spectrum to the F_2 -axis is shown. On the left hand side, the projection of the 2D-spectrum to the F_1 -axis is the ^1H -NMR spectrum. (20 mg of the substance were used in 0.5 mL D_2O . Data acquisition time was 15.4 hrs).

Figure 6.54 400 MHz (H, H) -COSY spectrum of neuraminic acid.

then the coupling nuclei may be difficult to identify.

If Θ_x is chosen to be smaller than 90°_x , the spectrum is simplified. The magnetization is transferred preferentially, such that some signals among the diagonal or correlation peaks are weaker than others. A smaller angle has the disadvantage that the sensitivity is reduced. A fair compromise is an angle of 45° (sometimes maybe 60°).

As an example we consider the 400 MHz (H,H)-COSY spectrum of neuraminic acid. Figure 6.54 shows the 400 MHz homonuclear (H, H)-COSY-45 spectrum of neuraminic acid in the region of $\delta = 1.4$ to 4.2 ppm. The section from $\delta = 3.4$ to 4.2 is depicted in enlarged form in Figure 6.55. Projections of the peaks to the F_2 -axis are given at the upper edge of the 2-D spectrum and the 1-dimensional ^1H -NMR spectrum is given on the left side of the 2-D spec-



Enlarged part of the spectrum of a neuraminic acid derivative. With the signals of protons H-4, H-7, and H-9' that are given in the upper 1D spectrum, it is possible to locate the signals of H-5, H-8, and H-9 by analysis of the correlation peaks.

Figure 6.55 Enlarged section of the homonuclear (H, H)-COSY-45 spectrum of neuraminic acid shown in Figure 6.54

trum. To analyze the 2-D spectrum, we connect the diagonal and the crosspeaks (correlation peaks) to form squares. Beginning with the unambiguous assignments of the H-3a and H-3e protons, the correlation peaks can be used to find the chemical shifts of the neighbor protons, which is the H-4 multiplet at $\delta = 4.0$ to 4.05 . This multiplet forms the corner of another square (Figure 6.55), by which the chemical shift of H-5 can be identified. Since the difference of the chemical shifts between H-5 and H-6 is small, it is difficult to determine the corresponding square. The analysis is preferably continued with another unambiguous signal, H-7, which shows a doublet at $\delta = 3.6$. The correlation peaks lead to a coupling nucleus, which can be H-6 or H-8. We cannot easily distinguish between these nuclei. However, it is known by other experiments that the coupling constant $J(\text{H-6, H-7})$ is small. The correlation peak therefore points to H-8. From H-8 we can find the shift of H-9' and H-9.

The example demonstrates that we need some clearly assigned peaks to start the interpretation of the 2-D (H, H) COSY experiment. In this example, the assignments of the peaks of H-3 and H-7 were used as a start. The signals of the other protons could be identified with the exception of some uncertainty in the section of $\delta = 3.85$ to $\delta = 3.95$, since in this region the multiplets of H-5, 6, 8, 9, and the methyl signal of the ester are superimposed. Another result is that non-coupling protons, in this case those of the methyl groups, only exhibit signals on the diagonal.