Electron Paramagnetic Resonance

Plan

(1) Principle of the EPR method
  (a) history of the EPR method discovery
  (b) mechanic and magnetic moments of electron
  (c) Zeeman's effect
  (d) resonance basic equation
(2) EPR spectra characteristics
  (a) amplitude
  (b) line shape
  (c) line width
  (d) superfine structure
(3) Scheme of an EPR radiospectrometer
(4) EPR application in medical biological investigations
  (e) natural EPR signals observed in biological systems
  (f) the method of spin labels and probes
  (g) the method of spin traps

Principle of the ERP method

History of the EPR method discovery

The EPR method is a basic method for investigation of paramagnetic particles present in biological systems. Two main types of compounds can be assigned to paramagnetic particles present in biological systems — these are free rsdicals and transit valency metals such as Fe, Cu, Co, Ni, Mn, or their complexes. Besides free radical states the EPR method can be applied for investigation of triplet states arising in the course of photobiological processes.

The method of electron paramagnetic resonance was discovered in 1944 in Kazan University by Evgeniy K. Zavoyskiy in the course of investigations of electromagnetic energy absorption by paramagnetic metal salts. He noticed that CuCl2 monocrystal put to a stationary/static (constant) magnetic field of 40 Gauss (4 mtl) started to absorb microwave radiation with a frequency of about 133 MHz. The pioneers of EPR application in biological studies in the USSR were L. A. Blumenfeld and A. E. Kalmanson who published in BIOFIZIKA journal in 1958 an article about the investigation of free radicals obtained under the action of ionizing radiation on proteins.

Mechanic and magnetic moments of an electron

Electron orbital and spin motion s lie in the basis of their orbital and spin mechanic moments. Electron spin magnetic moment is associated with electron spin rotation that can be presented as a motion around its own axis. Electron spin mechanic moment is (5.9)

$$P_s = \frac{\mathrm{h}}{2\pi}\sqrt{S(S+1)}\,, \quad (5.9)$$

where S is spin quantum number equal to 1/2.

Magnetic and mechanic spin moments are related as follows (5.10)

$$\mu_S = -P_S\,\frac{\mathrm{e}}{\mathrm{m}} = M_S\,\frac{\mathrm{eh}}{2\pi\mathrm{m}}, \quad (5.10)$$

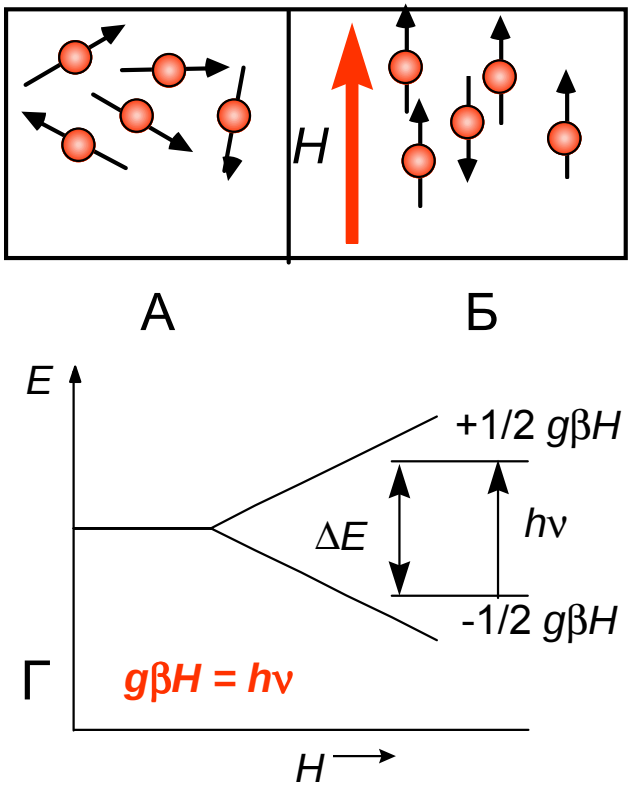
where M_s is magnetic quantum number equal to ± ½. The relation between the magnetic and mechanic moments is called gyromagnetic ratio (g). It can be seen that $y = -e/2m$ for the orbital motion, and $y = -e/m$ for the spin motion.

For gyromagnetic relation of electrons having different contribution of orbital and spin motion, a proportionality coefficient (g) is introduced, such as

$$\gamma = -g\frac{e}{2m} \tag{5.11}$$

This proportionality coefficient is called g-factor; $g = 1$ at $S = 0$, i. e. the electron spin motion is absent while only the orbital motion is present; and $g = 2$ if the orbital motion is absent and only the spin one is observed (for example, for free electron).

Zeeman's effect



In the absence of external magnetic field, electron magnetic moments are oriented (directed) casually, and their energies do not actually differ from each other (E0). When an external magnetic field is applied, electron magnetic moments become oriented (directed) towards the field, depending on the value of spin magnetic moment, and their energetic level splits (divides) into two (E_s= + 1/2, and E_s= – 1/2).

The energy of interaction between electron magnetic moment and magnetic field can be expressed by the followinf equation

$$E = -\vec{\mu}\,\vec{H} = -\mu H\cos(\vec{\mu}\,\vec{H}) \tag{5.12}$$

It follows from Eq.5.11 that

$$\mu_S = -gP_S\frac{e}{2m} = -gS\frac{he}{4\pi m} = \pm\frac{1}{2}g\beta \tag{5.13}$$

and the energy of interaction between electron and external magnetic field will be expressed as follows

$$E = \mu_S H = \pm\frac{1}{2}g\beta H \tag{5.14}$$

In this case the difference in energy between two levels will be

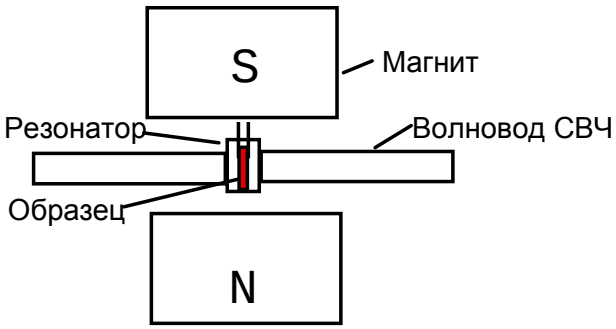
$$\Delta E = g\beta H \quad (5.15)$$

Eq. (5.14) describes Zeeman's effect that can be expressed by the following words: the energetic levels of electrons applied to magnetic field split in this field depending on the value of spin magnetic moment and the intensity of magnetic field.

The number of electrons having this or that energy will be determined in accordance with the Boltzmann distribution

$$\frac{n_{1/2}}{n_{-1/2}} = e^{-\frac{\Delta E}{kT}} = e^{-\frac{g\beta H}{kT}} \quad (5.16)$$

where n1 and n2 are the number of electrons on a higher or lower energetic level corresponding to the magnetic moment of electron with +1/2 or –1/2 spin.



If now electromagnetic energy is applied to the system of electrons existing in the magnetic field, electron transitions between levels will occur at some (definite) values of the energy of incident quantum. The necessary condition of the transitions is the equation of the incident quantum energy (hν) to the difference of energies between the levels of electrons with different spins (gBH)

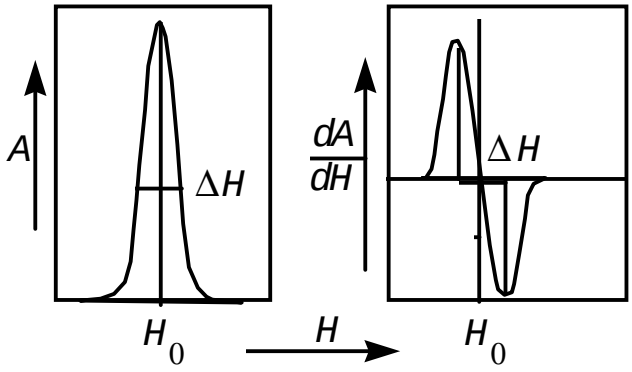
$$\Delta E = h\nu = g\beta H \quad (5.17)$$

Eq.(5.17) expresses the basic condition of energy absorption by electrons. Under the action of radiation, the electrons of (on) a higher energetic level will be emitting energy and returning onto a lower level. This phenomenon is called «induced emission». Meanwhile, the electrons on the lower level will be absorbing the energy and passing onto a higher energetic level. This phenomenon is called «resonant absorption». As far as the probabilities of single transitions between energetic levels are equal, and general probability of the transitions is proportional to the number of electrons at a given (definite) energetic level, the absorption of energy will prevail over its (the) radiation. It is associated with the fact that, as it follows from Eq.(5.16), the population of a lower level is higher (larger) than that of a higher energetic level.

EPR spectra characteristics

Signal amplitude

EPR signal represents the first (-order) derivative of absorption line. The area under the line of absorption is proportional to the concentration of paramagnetic particles in the sample. Thus, the concentration of paramagnetic centers is proportional to the first integral under the line of absorption or to the second integral of EPR spectra



If two signals are of the same width, the concentrations of paramagnetic centers are related like the signal amplitudes. To determine concentration, it is necessary to measure the area under the absorption curve of the standard with a known paramagnetic center concentration, and that of the sample measured; the unknown concentration is found from the proportion (under the condition that both samples are of the same shape and volume)

$$C_{изм.} = C_{эт.} \frac{S_{изм.}}{S_{эт.}} \qquad (5.18)$$

where Cmeas. And Cst. Are concentrations of the sample measured and the standard, respectively; and Smeas. And Sst. Are the areas under the absorption lines of the measured signal and the standard.

Line shape

Although, according to the basic resonance equation, the absorption occurs only when incident quantum energy is equal to the energy difference between the levels of unpaired electrons, the EPR spectrum is not linear but continuous in the vicinity of a resonance point (at a resonance point).

Line width

The width of the EPR spectrum depends on the interaction between the magnetic moment of electron and those of the surrounding nuclei (grate/lattice) and electrons. T1 is the time of spin-grating relaxation; it characterizes mean spin state

Life-time

It follows from the ratio of the Heisenberg uncertainty that

$$\Delta E \geq \frac{\mathrm{h}}{2\pi} * \frac{1}{\Delta t} \qquad (5.32)$$

If t is considered (to be) equal to T1, and E corresponds to gB H, then Eq. (5.32) may be rewritten as follows (5.33), i. e. the uncertainty in the line width is inversely proportional to the time of spin-grating relaxation.

Besides the interaction of the magnetic moment of unpaired electron with the grate, its interaction with the magnetic moments of other electrons is also possible. This interaction results in a decreased time of relaxation and thus in a broadened EPR spectrum line.

$$\Delta H \geq \frac{\mathrm{h}}{2\pi g\beta} * \frac{1}{T_{эфф.}} = \frac{\mathrm{h}}{2\pi g\beta} * \frac{2T_1 + T_2}{2T_1T_2} \qquad (5.33)$$

Among the mechanisms of line broadening the following ones should be mentioned: dipole-dipole (doublet) interaction; g-factor anisotropy; dynamic broadening of the line; and spin exchange. Interaction between the magnetic moment of unpaired electron and a local magnetic field created by neighbouring electrons and nuclei lies in the basis of the «dipole-dipole interaction». The strength of magnetic field at a point depends on the distance to this point and mutual orientation of the magnetic moments of unpaired electron can be calculated by the following equation

$$\Delta E = h\Delta\nu = g\beta\Delta H = g\beta\frac{\mu}{R^3}(3\cos^2\theta - 1) \qquad (5.34)$$

where m — electron magnetic moment, R — distance to the source of local magnetic field, Q — angle between interacting magnetic moments.

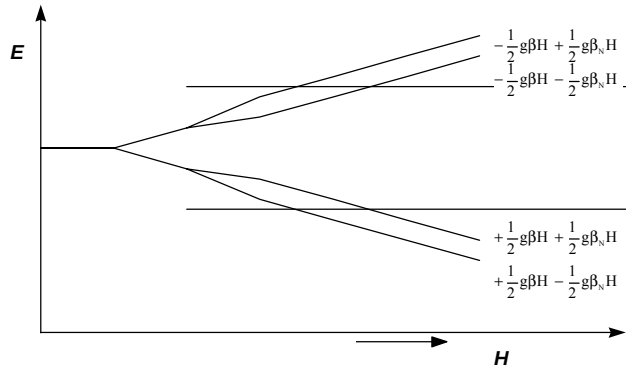
This interaction results in a deviation of the g-factor from the value 2.0023 that corresponds to free electron. For crystalline samples, g-factor values corresponding to crystal orientation are designated as gxx, gyy, and gzz, respectively.

Spin exchange is one more way of EPR signal spreading (broadening). The mechanism of signal spreading on spin exchange represents a change of the direction of electron's spin magnetic moment for the opposite one when impacting with another unpaired electron or some other paramagnetic. Since in such a collision the life-time of electron in this state reduces, the EPR signal broadens. EPR line broadening by the mechanism of spin exchange is most often signal broadening in the presence of oxygen or paramagnetic metal ions.

Superfine structure

The phenomenon of superfine interaction (i. e. interaction of the magnetic moments of unpaired electrons (M5) with those of nuclei (Mn)) lies in the basis of EPR line splitting into several lines.

Since in the presence of nucleus' magnetic moment the summary magnetic moment is (equal to) Ms+Mn (where Ms — electron's magnetic moment, Mn — nucleus' magnetic moment), summary magnetic field Hsum.=Ho+-Hloc., where Hloc. Is local magnetic field created by nucleus magnetic moment.

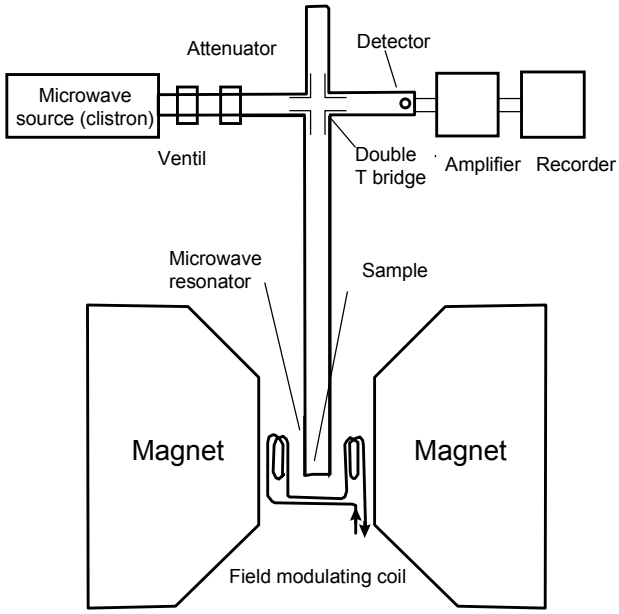


In this case there will be two resonance values instead of one — Ho+Hloc. and Ho-Hloc., and two lines will be corresponding to them. Thus, we have two lines at Ho+Hloc. and Ho-Hloc. instead of one at Ho.

A very alike (similar) pattern is observed when one unpaired electron interacts with several equivalent (with similar constant of superfine interaction) nuclei with a magnetic moment differing from zero, for example, with two protons. Three states corresponding to the orientation of proton spins arise in this case: (a) both along field, (b) both against field, and (c) one along field, and the other against field. The third variant (c) is twice more probable than variants (a) and (b) since it can be performed by two ways. As the result of such a distribution of unpaired electrons, single line will split into three lines with an intensities ratio of 1:2:1. In general case, the number of lines is 2nMN+1 for the number (n) of equivalent nuclei with the spin MN.

Scheme of an EPR radiospectrometer

The source of radiation in radiospectrometer is a klystron which represents a radiolamp giving monochrome radiation (with) in the cm-wave band.



The cuvette in radiospectrometer containing the sample is in a special unit called resonator. Its shape and parameters are such that standing wave is formed in it. Radiation, having passed the sample in the radiospectrometer and spectrophotometer, gets onto the detector, the detector signal then being amplified and recorded on a recorder or computer. The radioband radiation is transmitted from the source to the sample and then to the detector with the use of special rectangle section tubes that are called waveguides.

The dimensions of waveguide sections are determined by transmitting radiation wave-length. Constant radiation frequency is used in radiospectrometer for EPR spectrum recording, and the condition of resonance is achieved by changing the magnitude of magnetic field.

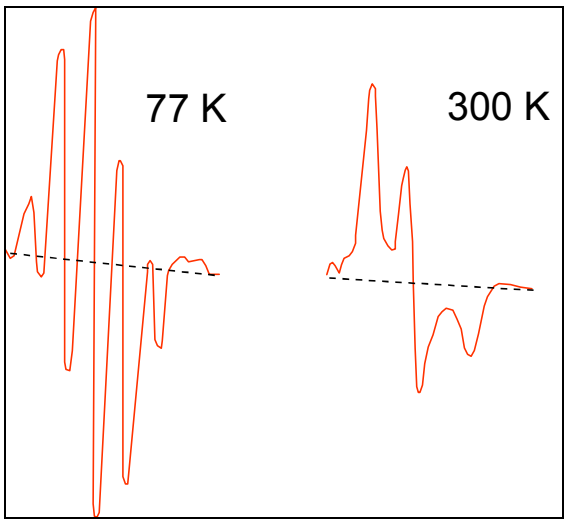
Amplification of the signal by way of its modulation by high-frequency variable field is one more important feature of radiospectrometer. As the result of modulation of the signal, its differentiation occurs, and absorption line transforms to its first derivative which is the signal of EPR.

Application of EPR in medical biological investigations

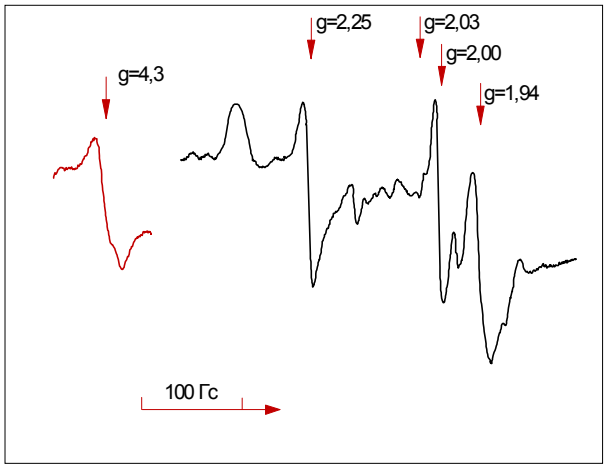
Natural EPR signals observed in biological systems

Application of the EPR method in biological investigations is associated with studies on paramagnetic centers of two basic types — free radicals and transit valency metal ions. Investigation of free radicals in biological systems is associated with a difficulty that lies in a low concentration of free radicals that are formed in the process of cell life-time. Radical concentration in normally metabolizing cells is, according to different sources, about 10—8 to 10-10 M, while modern radiospectrometers allow to measure radical concentrations of 10-6 to 10-7 M. Concentration of free radicals can be increased by inhibition of their death and increase of their formation rate. This can be done by way of irradiation (UV- or ionizing radiation) of

biological objects at low temperature. It was the investigation of the structure of radicals of more or less complex biologically important molecules that was one of the first directions of application of the EPR method in biological investigations.

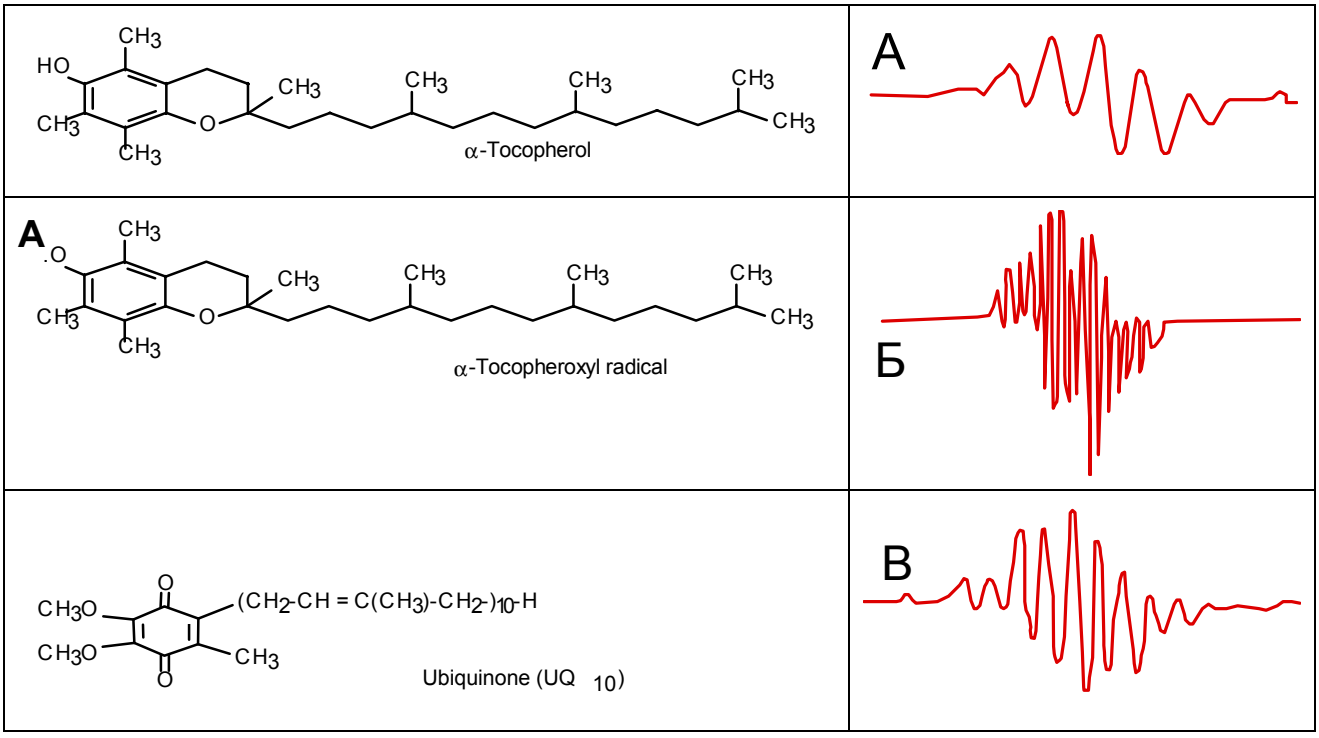


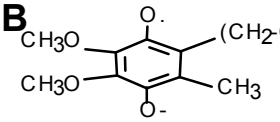
EPR spectrum of UV-irradiated cystein at 77 K



EPR spevtrum of rat liver at 77 K

Another important direction of the EPR method application in biological investigations was investigation of transit valency metals and/or their complexes existing in vivo. While looking at the EPR spectrum of, for instance, rat liver one can see cytochrome P-450 signals with g-factors 1.94 and 2.25; methemoglobin signal with g-factor 4.3; and free-radical signal belonging to semiquinone radicals of ascorbic acid and flavins with g-factor 2.00. Due to short-time relaxations, the EPR signals of metal proteins can be observed only at low temperatures, for example, at liquid nitrogen temperature. However, the EPR sugnals of some radicals can be observed at room temperature. Such signals include the EPR signals of many semiquinone or phenoxyl radicals such as semiquinone radicals of ubiquinone, phenoxyl, and semiquinone radical of a-tocopherol (vitamin E), vitamin D, and many others.



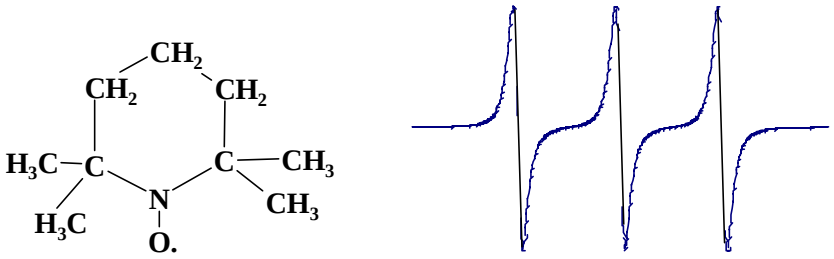
B  Ubisemiquinone radical (USQ $_{10}$)	

The method of spin labels and probes

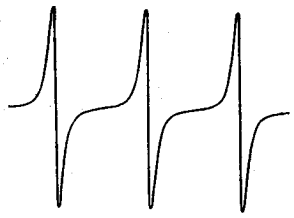
The synthesis of stable free radicals became an important stage in the development of application of the EPR method in biological investigations. Among such radicals, nitroxyl radicals became the most popular. The stability of nitroxyl radicals is conditioned by space screening of >N-O· group that has unpaired electron, and by four methyl groups inhibiting the process of reaction with the participation of free valency.

Such screening is not, however, absolute, and the reaction of free valency reduction can nevertheless occur. Ascorbic acid, for example, is a good reducer of nitroxyl radicals.

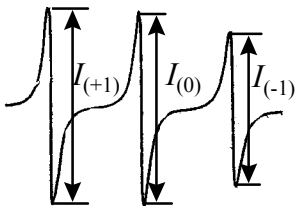
The EPR spectrum of nitroxyl radicals consists, in a simplest case, of three lines of equal intensity due to the interaction of unpaired electron with nitrogen atom nucleus having whole-number spin +-1 and 0.



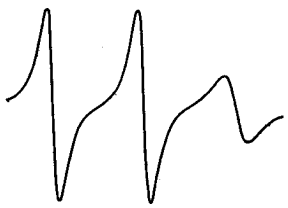
Chemical structure and the EPR spectrum of the spin probe TEMPO

$3 \cdot 10^{-12}$ s 

If nitroxyl radical is in water solution its spinning is isotropic and rather speedy which results in averaging of the EPR spectrum anisotropy.

$1 \cdot 10^{-10}$ s 

Anisotropic interactions appear at a decreased spinning rate which leads to broadenning of the lines and, correspondingly, to changes of the amplitudes of spectrum components, and then to a shift of edge components.

$1 \cdot 10^{-9}$ s 

The notion «correlation time» (T_s) is used for description of radical motion. This time is the time of turning of free radical by an angle of 90^o.

For correlation times within the range from $5 \cdot 10^{-11}$ to 10^{-9} s the following formula is used

$$\tau_c = 6{,}65 * \Delta H_{+1}(\sqrt{\frac{I_{+1}}{I_{-1}}} - 1) * 10^{-10} \text{сек}, \tag{5.35}$$

where H_{+1} is the width of the low-pole component of the EPR spectrum; I_{+1} and I_{-1} are the amplitudes of the high-pole and low-pole lines. At slower motions of nitroxyl radical molecule ($T_s > 10^{-9}$ sec) correlation time can be measured by the formula

$$\tau_c = a(1 - S)^b \tag{5.36}$$

where a and b are the parameters that depend on radical type and motion model, and

$$S = \frac{A'_{zz}}{A_{zz}} = \frac{H_{-1} - H_{+1}}{H_{-1\max} - H_{+1\max}} \tag{5.37}$$

where $2A_{zz}$ and $2A'_{zz}$ is the distance between the high-field and low-field components of the EPR spectrum in the given spectrum ($2A_{zz}$) and at the maximally long correlation time (2Azz'), and H_{-1}, H_{+1}, $H_{-1\max}$, $H_{+1\max}$ are the values of magnetic field strength corresponding to these spectrum components. The correlation time of nitroxyl radical depends immediately on the medium microviscosity. Using the Stocks-Einstein equation, it is possible to determine viscosity in a corresponding macroscopic system

$$\eta = \tau \frac{3kT}{4\pi R^3} \tag{5.38}$$

where R — nitroxyl radical effective radius. It is seen from Eq. (5.38) that, using nitroxyl radical as a probe, it is possible to determine the microviscosity of radical-containing medium. This technique is widely used in biological investigations for measuring the microviscosity of biological membranes.

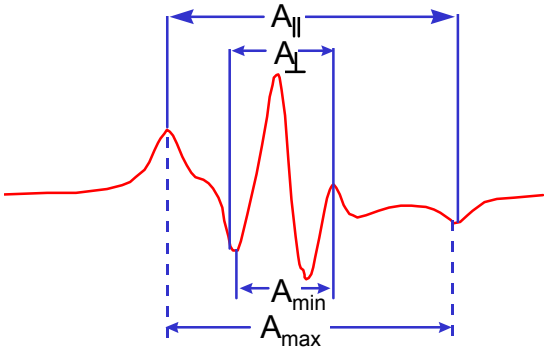


Fig.. EPR spectrum of 5 doxylstearate

The above described probe behaviors and EPR spectrum changes are true of spin-probe isotropic rotation in a homogeneous medium such as water or glycerol solution. Nevertheless, if the character of probe rotation changes (for example, rotation around one of the spin probe axes prevails), then the EPR spectrum also changes. Changes in the character of spin probe rotation can occur if the probe represents a fragment of some other molecule, for example, that of fatty acid present in the membrane. If spin probe is attached to the fifth atom of stearic acid molecule present in the membrane it will be rotating mostly around the long axis of fatty acid molecule, i. e. rotation anisotropy appears. This will naturally be reflected on EPR spectra. To describe spin probe rotation anisotropy, the ordering parameter (S) is used.

$$S = \frac{A_{\parallel}^{'} - A_{\perp}^{'}}{A_{zz} - \frac{A_{xx} + A_{yy}}{2}} \qquad (5.39)$$

where $A_{\parallel}$' and $A_{\perp}$' are the parameters indicated in Fig. 12. The order parameter equals to 1 if the probe rotates around the normal to the membrane. On membrane dilution, the cone of revolution will be widening, and the value of order parameter (*S*) will be approaching zero. Thus, the order parameter alters from 0 to 1 with an increase of membrane viscosity and structurization. This property of fatty-acid spin probes allows to use them as detectors for measuring viscosity and anisotropy of membrane structure.

The natural sequence of application of fatty acid-containing spin probes is the possibility of measuring the ordering parameter at different distances from membrane surface, the so-called ordering profile, or viscosity profile. Such measurements appear to be possible when using a set of homogeneous spin probes each containing nitroxyl fragment at a different distance from carboxyl group. For example, spin probes with nitroxyl radical at the 5^{th}, 7^{th}, 12^{th}, and 16^{th} carbon atom of stearic acid are now widely used. A set of these compounds allows to measure the S parameter at a distance of 3.5, 5.0, 8.5, and 10.4 Å from membrane surface.

As it was shown above, the EPR spectra of spin probes differ very much from each other; this depends on the fact whether the spin probe is in a viscous or nonviscous surrounding, for example, in water or in membrane. This property of spin probes was used for creation of new-class spin probes allowing to measure membrane'' surface potential. To measure membrane surface potential, the coefficient of water/membrane distribution of neutral and charged probes is measured. Since charged probe interacts with the charges on the membrane surface, its distribution coefficient will differ from that of neutral probe. A ratio between distribution coefficients serves the measure of the given membrane surface potential. This value can be standardized (normalized) by measuring the distribution coefficients of spin probes in the membranes that have the highest positive and negative potentials. The EPR spectra of the spin probes used for measuring surface potentials are shown below.

Using spin probes that interact specifically with sulfhydryl groups, it is possible to determine easily SH-group concentration in various proteins. The scheme of spin probe interaction with sulfhydryl groups is given in the following figure (Fig.15).

Everythig said above in the section about practical application of nitroxyl radicals concerned the method of spin probes. Nevertheless, the method of spin labels is no less perspective. This methos is also based on the principle of changing the EPR spectrum of nitroxyl radical depending on the rate and isotropy of its rotation. The method differs only by the fact that a spin label binds covalently with another more or less large molecule or macromolecule. One of the first and successful applications of the spin-label method was the measurement of the amount and accessibility of protein SH-groups. The schematic formula and EPR spectrum of the spin label interacting with SH-groups in free state and after its attaching to protein are given in Fig.16. It can be seen from the figure that the EPR spectra of the spin label in free and bound state differ very much; it is associated with the difference in the rate and direction of rotation. It is natural that a bound spin label has a significantly lower rotation than in free state. Moreover, the quantity of bound spin labels and, correspondingly, the intensity of EPR signal is proportional to the quantity of SH-groups that reacted with the spin label. The mobility of the spin label attached tj protein can also change in the case if the protein possesses fermentative (enzymatic) activity. The mobility of the spin label located near enzyme's active center can significantly change during the interaction of the enzyme with substrate.

An analogous method based on inhibition of nitroxyl radical rotation can be also used when studying the the binding of spin-labeled enzyme substrates or other biologically important compounds with macromolecules and membranes. In this case the EPR spectrum will change only when the substrate under study is closely bound with enzyme or built-in to membrane. There presently exist a lot of methods allowing to investigate protein globule topography using spin labels. The most widespread methods are the following: (1) «Spin label — paramagnetic metal ion». Since many transit valency metal ions are paramagnetic and can besides be found in enzyme's active center, the interaction with the ion of the spin label attached, for example, to cysteine or hystidine residue of protein globule will lead to EPR spectrun broadening as the result of the dipole-dipole interaction of paramagnetics; (2) «Spin label — spin label». An analogous effect can be brought about by two spin labels that stand in the immediate vicinity to each other if they are attached to corresponding protein groups. In this case, if during fermentation the distances between these labels change, this will be easily seen on the EPR spectrum; (3) «Spin probe — spin label». It is possible to determine the distance from the surface of protein globule to the location site of spin label by adding the spin probe that does not penetrate between the coils of protein a-spiral to the protein containing spin label. In this case the interaction between spin label and spin probe will lead to changes in the EPR spectra.

The method of spin traps

The appearance of nitroxyl radicals became a decisive event for solution of the problem of detection and investigation of free radicals forming in living systems. The detection of radicals became possible due to the introduction of the method of spin traps. The essence of the method consists in the following: a compound that is no nitroxyl radical but has a srtucture close to nitroxyl radical (spin trap) interacts with a free short-life radical and transforms to a long-life nitroxyl radical (spin adduct) whose EPR spectrum is unique for the given radical or family of radicals. The scheme of the reaction of spin trap C-phenyl-N-tret-butyl nitron with hydroxyl radical is presented in Fig.17.

By their chemical nature, spin traps can be attached to two basic classes — nitrons and nitroso compounds. C-phenyl-N-tret-butyl nitron (PBN), as it follows from its name, is attached to nitrons. One more popular spin trap is 5,5-dimethyl-pirrolin-1-oxyl (DMPO) that is also attached to nitrons. Among nitrosocompounds, nitrosobenzene and nitrosodurene tret-nitrosobutane should be mentioned. The chemical formulae and schemes of reactions are presented in Fig.18.

The presented spin traps differ from each other by the rate of reaction with radicals, stability of formed spin adducts, and values of differences in the spectra of spin adducts for different radicals. Presently, there already exist data banks containing parameters of the EPR spectra of spin adducts which allow to precisely identify and determine free radical concentration by the EPR spectrum of its spin adduct.