Topics in Applied Chemistry

Electron Paramagnetic Resonance in Biochemistry and Medicine

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Preface

Scientific and technical progress contributes a great deal to the development of medical science. During the last years the essence of many pathogenic mechanisms of various diseases has been discovered and new effect methods of their diagnosis and treatment have been developed.

Pathologic states are caused by not only disorders of lipid, hydrocarbon, protein and mineral metabolism. Alterations in redox processes are also of importance. Paramagnetic centers (PMC) presented in many biological tissues and fluids can serve as indicators of these changes owing to the presence of free radicals and cations of transition microelements. These centers presented in different organic and inorganic biomolecules are cofactors of some enzymes, which provides a valuable, and sometimes unique, information concerning qualitative and quantitative changes in biological objects.

This book demonstrates the unlimited possibilities of the application of Electron Paramagnetic Resonance (Electron Spin Resonance) spectroscopy in biochemistry and medicine. We use the term Electron Paramagnetic Resonance (EPR) spectroscopy in the book.

EPR spectroscopy is an objective and accurate method of recording paramagnetic centers widely accepted in chemistry, biochemistry, biology and medicine.

The EPR method was discovered in 1944 by Eugene Konstantinovich Zavoisky in Kazan. In the middle fifties, nearly simultaneously in the United States and the Soviet Union, EPR spectroscopy was first used for the investigation of tissues and fluids in human and animals. In that time, owing to low sensitivity of EPR spectrometers, the method of tissue lyophilic drying was used. The development of low-temperature registration of

biological tissues in the early seventies extended the scope of application of EPR spectroscopy in biochemistry and medicine. By the present time a high sensitivity and precision of this method has been achieved, which allows investigation of even native water-containing tissues.

At first, the use of EPR spectroscopy in biochemistry and medicine was aimed at studying malignant tumors. The synthesis and introduction of spin labels, probes and traps made it possible to discover many mechanisms of pathologic states. During the last years the possibility of using EPR spectroscopy *in vivo* has been intensively examined.

The literature data devoted to the investigation of paramagnetic centers in human substrates and to the possible practical use of the results, are scarce, dissipated and non-summarized. This monograph is an attempt to fill this gap and to give an outline of pathways and prospects for the use of EPR spectroscopy in medicine and biochemistry.

Practically all publications dealing with the use of EPR spectroscopy in medicine and biochemistry and the authors' own results on EPR investigation of biological fluids from the organism of normal individuals and patients with different pathologies have been summarized and analyzed in the present monograph.

The EPR spectra of blood and blood components, saliva, nasal secretion, synovial fluid, gastric and duodenal contents, feces, teeth, bones and some other tissues and biological fluids have been considered in this book. The quantitative and qualitative composition of paramagnetic centers in normal individuals and patients suffering from different diseases is presented.

Special attention is given to the EPR examination of bio-molecules (enzymes, polypeptides, vitamins, lipids, hydrocarbons, etc.) playing an essential role in the vital activities of man. Also the possible use of this method for the investigation of drugs, toxicants and metabolites as well as food products is discussed. There is a section deals with the use of EPR spectroscopy in dosimetry and diagnosis of irradiation injury in the book.

Fundamental problems related to the study of mechanisms of the development of oncologic, cardia-vascular and other diseases including AIDS have been elucidated. Much attention is given to the use of the EPR method diagnostics, investigation of metabolism, free-radical reactions in the development of pathologies, as well as in EPR-dosimetry and archaeology. EPR technique extends our concepts concerning the role of transition microelements in human activities and in fine mechanisms of pathogenic processes occurring in various diseases. This method also opens up fresh approaches to new therapeutic and prophylactic measures.

Special attention in the monograph is given to the application of EPR spectroscopy for a better understanding of pathogenesis of diseases, diagnosing and choosing the proper methods of treatment.

The monograph contains a large volume of EPR-derived information on biological processes occurring in the human body, such as generation of oxygen radicals ("respiratory oxygen burst"), nitric oxide metabolism, *etc.* It also considers the possibility of using spin labels and spin probes for the diagnosis of diseases and scavenging (trapping) of free radicals of various origin. The potential uses of the EPR method in radiation medicine, stomatology, pharmacology, archaeology, and food industry are discussed.

These above studies are updated with the literature data on EPR investigations in the field of biochemistry and medicine, published by 1999.

The monograph is intended for physicians, surgeons, otorhino-laringologists, and doctors of other specialities interested in recent problems in medicine. It will undoubtedly be of help for biologists, biochemists, biophysicists and chemists engaged in ESR spectroscopy. The book can be useful for students at the senior undergraduate or graduate level either as a textbook in a course or as a self-study guide. To everyone who is interested in modem problems in medicine, biochemistry, biophysics, ecology, and cares about his health and food.

All additions, recommendations and comments of the readers will be appreciated.

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Contents

1. ELECTRON PARAMAGNETIC RESONANCE (EPR): THEORY AND METHOD 1
1.1 FUNDAMENTALS OF THE EPR THEORY 1
1.1.1 Integral Intensity 3
1.1.2 Line Width 3
1.1.3 g-Factor 4
1.1.4 Hyperfine Structure and Its Origin 5
1.1.5 Saturation Effect 5
1.2 PROCEDURES FOR STUDYING PARAMAGNETIC CENTERS 6
1.2.1 Preparation of Samples 6
1.2.2 Thermostatic Control of Frozen Samples 8
1.2.3 EPR Spectra Recording 9
1.2.4 Standardization of Measurement Conditions 12
1.2.5 Determination of PMC Concentration in Biological Fluids Isolated from the Human Organism 14

REFERENCES 17

2. PARAMAGNETIC CENTERS IN THE HUMAN BIOLOGICAL MEDIA 21
2.1 ERYTHROCYTES 21
2.2 BLOOD PLASMA 34
2.3 SALIVA 43
2.4 NASAL SECRETION 46
2.4.1 Free Radicals 47
2.4.2 Nasal Secretion Metal-Proteides in Volunteers 48
2.5 TEETH 50
2.6 CONTENTS OF STOMACH 51
2.7 DUODENAL CONTENTS 55
2.8 SYNOVIAL FLUID 60
2.9 FECES 62

REFERENCES 68

3. PARAMAGNETIC CENTERS IN THE ORGANISM OF VOLUNTEERS AND PATIENTS IN VARIOUS PATHOLOGICAL STATES 75
3.1 BIOLOGICAL SUBSTRATES OF NORMAL INDIVIDUALS 75
3.2 ISCHEMIC HEART DISEASE 80
3.3 HYPERTENSIVE DISEASE 105
3.4 BLOOD DISEASES 110
3.5 HEMOSTASIS DISORDER 116
3.6 DISEASES OF GASTRO-INTESTINAL TRACT 124
3.7 LUNG DISEASES 137
3.8 DISEASES OF KIDNEYS 141
3.9 DIABETES 148
3.10 NERVOUS SYSTEM DISEASES 152
3.11 DISEASES OF JOINTS AND CONNECTIVE TISSUE 161
3.12 MYOGLOBIN PATHOLOGY 164
3.13 CUTANEOUS DISEASES 165
3.14 GYNECOLOGIC DISEASES 167
3.15 ONCOLOGIC DISEASES 170
3.16 OPHTHALMIC DISEASES 178
3.17 OTORHINOLARYNGOLOGIC DISEASES 181
3.17.1 Nasal Secretion Free Radicals 181
3.17.2 Nasal Secretion Metalloproteins 183
3.18 DISEASES OF TEETH AND MOUTH CAVITY 186
3.19 RADIATION-INDUCED PATHOLOGY 187
3.20 HYPERTHERMIA IN PATHOLOGIC STATES 195
3.21 HYPERBARIC OXYGENATION 196
3.22 CONDITIONS UNDER NARCOSIS 198
3.23 PATHOLOGIC STATES CAUSED BY INTOXICATION 199
3.24 SEPSIS-INDUCED STATES 201
3.25 PERITONITIS 202
3.26 ACQUIRED IMMUNODEFICIENCY SYNDROME (AIDS) 205

REFERENCES 206

4. OTHER ASPECTS OF EPR INVESTIGATION OF BIOLOGICAL SPECIMENS 239
4.1 FOOD PRODUCTS 239
4.2 DRUGS 241

4.3 FORENSIC MEDICAL EXAMINATION 245
4.4 ARCHAEOLOGY 245

REFERENCES 246

CONCLUSION 251

ABBREVIATIONS 257

Index 259

Chapter 1

ELECTRON PARAMAGNETIC RESONANCE (EPR): THEORY AND METHOD

1.1 FUNDAMENTALS OF THE EPR THEORY

Paramagnetic centers (PMC) are the sites in molecules and atoms having free unpaired electrons (UE). The method of electron paramagnetic resonance (EPR) is based on the possibility of recording UEs in atomic, crystalline ionic and molecular structures; in compounds oftransition metals (copper, vanadium, chromium, molybdenum, manganese, iron, nickel, *etc.*); upon cleavage of covalent chemical bonds. In the latter case UEs appear either at unoccupied atoms or at molecular and macromolecular fragments, *i.e.* at free radicals (FR) of higher activity. Many of the above electronic states have been revealed in biological objects.

The theory of EPR and its specific use in physics, chemistry, biology and medicine have been considered in many monographs and reviews [1-19]. During the last years the EPR technique has been constantly improved [20-22] and EPR *in vivo* investigations have been extensively developed [23-27]. EPR spectroscopy enables UEs to be detected and characterized. This is possible due to the fact that odd electrons have a magnetic moment caused by spin (S) equal to 1/2 (intrinsic moment of momentum or angular moment).

In order to characterize several UEs the general coordinate axis is needed. However, there is no axis of this kind in the absence of external magnetic field. UE spins are oriented in a random way. If the external

magnetic field is applied to the object to be examined a general coordinate axis would arouse around which all the electrons begin precessing in such a way that M_s, i.e., their spin projection on the axis, would be equal to either +1/2 or to -1/2 (Figure 1-1).

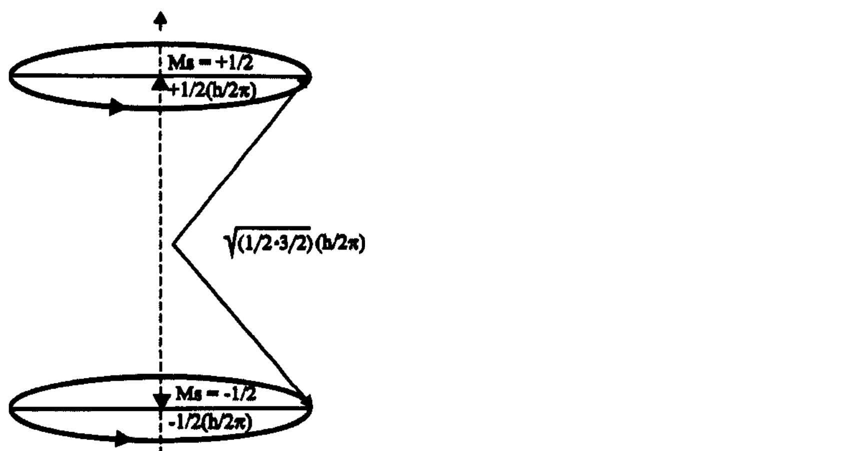

Figure 1-1. The projection of full magnetic moment on the quantum axis (the direction of the field).

In the external magnetic field UEs are subdivided into two groups: in one group the spins are oriented parallel and in the other antiparallel to the field direction. In the former the electron energy decreases and in the latter increases; this means that splitting of energy level occurs (Figure 1-2).

It is this phenomenon the EPR method is based on. When EPR spectroscopy is used the sample to be examined is placed into a strong homogeneous magnetic field and simultaneously exposed to electromagnetic radiation of a frequency with $h\nu$ (energy quantum) equal to the difference between the electronic levels of the two groups (the so called resonance frequency):

$$h\nu = g\beta H$$

Where

β is the Bohr magneton characterizing the ratio between the angular and magnetic moment,

g denotes a constant (g factor),

h stands for the Plank constant,

ν is the electromagnetic radiation frequency and

H is the magnetic field strength.

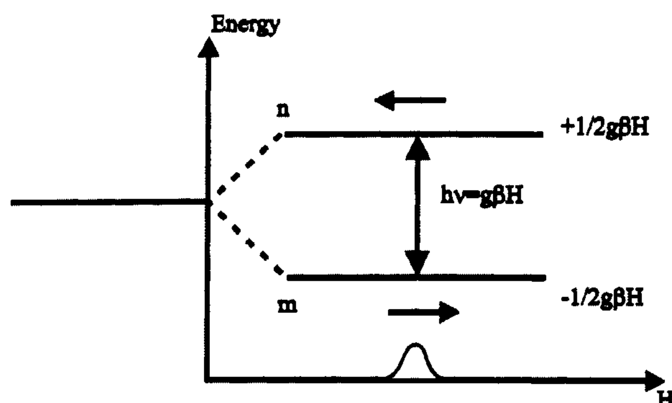

Figure 1-2. The main condition of electron paramagnetic resonance origin (under of the influence of the external magnetic field the energy levels of UEs are splitted).

Owing to radiation energy the odd electrons locating on the lower energy level pass onto the higher level with simultaneous change of the spin direction and energy absorption. The upper level electrons pass onto the lower level radiating a quantum of electromagnetic energy. This process is called "induced emission". The absorption and induced emission coefficients are equal. With the same number of electrons on the two energy levels there would be no absorption. In the reality, however, there is. This can be explained by the fact that the number of UEs on the lower energy level is always smaller than on the upper level. This difference is responsible for the observed EPR signal intensity. The latter presents one of the most important parameters characterizing the EPR spectrometer sensitivity.

Main parameters of EPR spectra are the following:

1.1.1 Integral Intensity

Integral intensity which is the difference in the energy level population and, consequently, the absorption value in the microwave region proportional to the total number of electrons in the sample. The concentration of electrons can be judged upon by the absorption line integral intensity.

1.1.2 Line Width

Two lines showing the same integral intensity may be of quite different form. The line width provides a supplementary parameter characterizing the absorption conditions and is determined by the scatter of energy levels

occupied with UEs. There are two major types of interactions giving rise to the resonance line broadening: spin-lattice and spin-spin interactions.

1. Spin-lattice interaction exists between the UE spin and its surrounding (crystal lattice or molecular system). In the present case the spin-absorbed energy is restored to the lattice or to the whole molecular system, which provides for continuous resonance absorption. Without this mechanism the electrons absorbing microwave energy would be passing from the lower to the upper level until their population would equalize and the absorption would cease. When the spin-lattice interaction is great the electrons can occupy the upper level for only a very short time. The strong interaction of this type causes a rapid spin-lattice relaxation thus leading to the absorption linebroadening.

2. Spin-spin interaction involves energy exchange in spins instead of giving energy to the crystal lattice or to the molecular system. Unlike spin-lattice interaction this does not favour thermal equilibration, arousing instead the resonance line broadening due to both direct spin interaction and a shorter life-span of spin states. This can be illustrated by normal dipole-dipole interaction with two bar magnets serving as an analog. Each UE interacts not only with the external magnetic field, but also with magnetic fields created by other UEs. At a high electron concentration in the sample the inter-electron space is small. This supplementary field can reach a fairly high value. In concentrated solutions of transition metal salts this value attains some scores of oersteds. In diluted solutions (including biological and biochemical systems) the UE content is much lower, so in this case odd electrons influence each other to a considerably less extent.

1.1.3 g-Factor

g-Factor characterizes the resonance point position. From the equation of resonance $h\nu = g\beta H$ it follows that with a constant microwave frequency the strength of externally applied magnetic field (H) and the g value are the only variable parameters. Consequently, the g-factor is determined by the field value giving rise to resonance. The g-factor of free electron (having no orbital moment) is 2.0023. In many FRs with UEs occupying a strongly delocalized molecular orbital the g-factors are very close to the above value which indicates a weak coupling of spin and orbital moments. The odd electron bonded to an atom of the crystal lattice is influenced by sufficiently strong internal electric field caused by the molecule structure. The g-factor value provides a detailed information on the character of intramolecular bonds.

1.1.4 Hyperfine Structure and Its Origin

Hyperfine structure (HFS) arises during the interaction between UE with the nuclear magnetic moment of the atom involved in the UE orbital. Nuclei of many atoms participating in biochemical processes (hydrogen, nitrogen, oxygen, sulfur, *etc.)* possess spin and magnetic moments. Owing to this fact the Zeeman levels are even more split (Figure 1-3).

According to the selection rule ($\Delta M_I = 0$), only the electron transitions with invariable spin nuclei orientation (I) are possible. With $I = \pm 1/2$ there are only two transitions of this kind. Correspondingly, in the EPR spectrum the absorption line is split into two components. When UEs are moving along molecular orbitals involving several atoms a hyperfine splitting caused by interaction of the electrons with different atoms occurs. In this case the spectral structure is very complex. In a general case of interaction with the nucleus spin $I = 1/2$ the number of HFS components is defined by the following formula: $2nI + 1$.

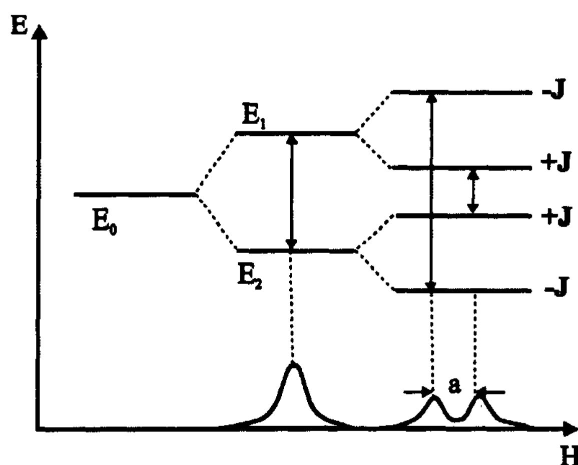

Figure 1-3. Hyperfine structure arising during the interaction between UE and nucleus with spin1/2.

1.1.5 Saturation Effect

As mentioned above, the EPR signal intensity is determined by the difference of the Boltzmann sublevel population. This difference exists due to relaxation phenomenon. With increasing the applied SHF (Super High Frequence) power a situation when relaxation would not be able to keep pace with equilibration is quite possible. As a result the population of the

upper and the lower levels would reach equilibrium and the EPR signal intensity would decrease correspondingly.

1.2 PROCEDURES FOR STUDYING PARAMAGNETIC CENTERS

In studying paramagnetic centers various EPR spectrometers are used - RE-1306, RE-1307 (Russia), "Bruker" (Germany), "Varian" (USA), "Jeol" (Japan), *etc.* In particular, the present authors made use of 3 cm range radio-spectrometers.

1.2.1 Preparation of Samples

The high potential of EPR spectroscopy in medicine became more evident with perfection of radiospectrometers, theoretical foundations of the method and ways of fixation of biological objects. In the first stages of investigation inadequate sensitivity ofthe instruments did not allow the EPR method to be used for studying aqueous biological systems. This made it necessary to use liophylic drying of tissues [4, 28, 29]. It turned out that the main contribution to the formation of FR signals of dried tissues is made by ascorbic acid [30-32]. Besides, a certain role is played by ubiquinone and other polyphenoles and quinones [33-35] The PMC of lyophilically dried tissues differ from those of aqueous tissues [36, 37]. Therefore it is desirable to examine tissues containing natural amounts of water.

The present authors used deep freezing at 77°K as a method of biological tissue fixation [38]. The EPR signal intensity depends on the PMC concentration, recording conditions and the sample size. So, only the samples of standard volume should be employed. The samples of this kind are made in teflon mold slightly modified molds [39] (Figure 1-4, sizes are gave in mm).

The mold consists of the body and the plug. Another procedure of preparing a sample to be analyzed on their spectrometer is proposed recently [40]. However, the drawback of this method is that cutter basis is made of silicone rubber containing vulcanizers, potential metal chelating agents. The reaction between them can greatly distort the EPR spectrum.

Blood for analysis was taken from the ulnar vein of patients at the same time in the morning (on empty stomach) and placed into an ampoule with heparin. Then the blood was centrifuged at 1500 rpm for 10 min. To precipitate the leukocytes the plasma was separated from the erythrocytes and centrifuged at 3000 rpm for 5 min. The erythrocytes were washed thrice with a cooled physiological salt solution (1:10 ratio).

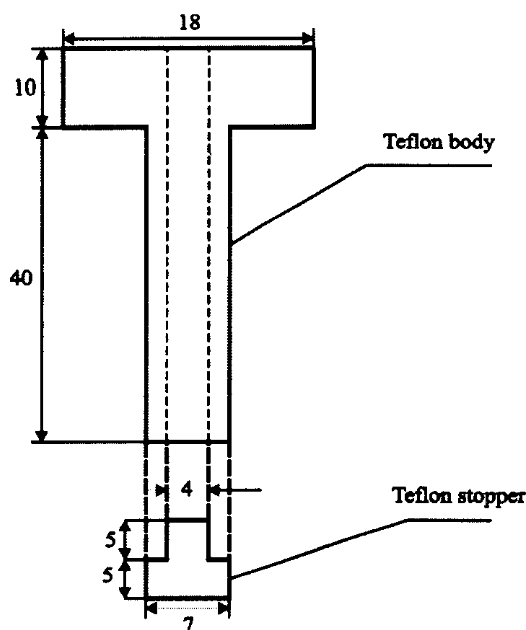

Figure 1-4. The teflon mold for the preparation of the samples at 77°K.

The synovial fluid was obtained by puncturing the joints for a diagnostic or therapeutic purpose.

The saliva was taken in the morning on the empty stomach for 20 minutes after a careful toilet of the oral cavity (without stimulation).

The nasal secretion was isolated just prior to sample preparation. For this purpose the use was made of a device the operation of which is based on mechanical stimulation of the nasal mucous membrane secretion and simultaneous NS aspiration from the surface of the superior and inferior turbinated bones and the nasal membrane into a sterile small flask. This precluded possible influence of blood, saliva and lachrymal fluid admixture to NS on the EPR spectroscopy results [15].

The device designed by the authors (Figure 1-5) consists of a metallic canula 125 mm long, Ø 0.7 mm, flexed in the distal and proximal sections at an angle of 120° with smooth end edges, a 15 ml flask with a ground stopper, an electro-sucker and connective tubes 3 mm in diameter.

The tube connecting the canula with the receiving flask is 150 mm long; the total length of the flask with the suction hose is 1170 mm. The above mentioned ratio are most appropriate for the NS aspiration from the nasal mucous membrane (NMM) under 0.2-0.4 kg/cm^2 vacuum produced by the sucker.

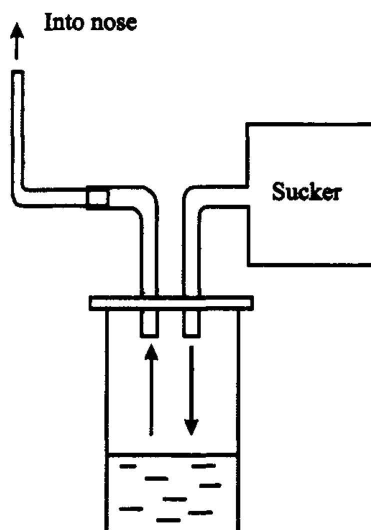

Figure 1-5. The device for isolating the nasal secretion that investigate it by EPR.

This device allows an atraumatic sighting NS aspiration from strictly defined NMM areas (visually controlled). Mechanical irritation of NMM in response to a careful touch of the canula distant end makes it possible to take necessary NS amounts even from patients with minimum secretory activity. The stomach contents were taken in the morning on an empty stomach by continuous vacuum aspiration.

Duodenal intubation was performed by a routine method in three batches (A, B and C).

The feces collected into small glass bottles were weighed in the mold before and after placement of the sample. Calculation was based on 100 mg of the substrate.

The samples prepared were frozen in liquid nitrogen, extruded from the mold, placed into quartz Dewar vessels and subjected to thermostatic control.

1.2.2 Thermostatic Control of Frozen Samples

The use of frozen samples provides much advantage. Freezing does not lead to any significant disruption of metabolic structures, and the EPR spectra of frozen samples are identical with those run at room temperature [3, 36]. When temperature drops the relaxation time of paramagnetic metal

complexes increases whereas the line width narrows thus enhancing the instrument sensitivity [9, 16-19].

However this method has certain shortcomings. At 77°K accumulation of oxygen (b.p. 92°K) takes place in the sample. Since oxygen is of paramagnetic nature a broad absorption signal displacing the zero line of the instrument appears in the EPR spectrum. Besides, constant boiling of liquid nitrogen in the Dewar vessel brings about the appearance of additional noises in the spectrum. The above drawbacks can be remedied by means of thermostatic control. For this purpose the liquid nitrogen boiling level was removed outside the resonator (cavity) (Figure 1-6) in the following way. The Dewar vessel containing the sample was cooled with liquid nitrogen which was poured out 30-60 sec later. The sample was covered with a layer of cotton 3-5 mm thick and again poured over with liquid nitrogen. As a result boiling occurred above the cotton tampon, *i.e.,* outside the resonator operating zone. Under these conditions the temperature in the lower part of the sample was only 6°C higher than that of the liquid [39]. After 1-2 min no temperature fluctuations are observed.

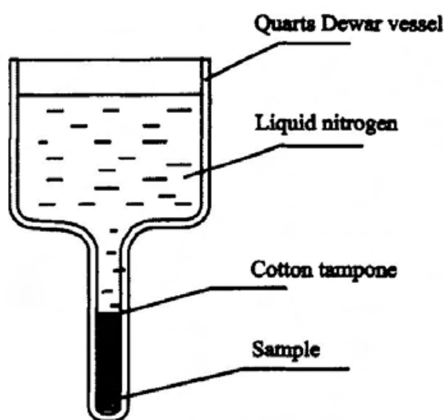

Figure 1-6. Scheme of the sample thermostatic control in the Dewar vessel at 77°K.

Thus, thermostatic control brings the liquid nitrogen boiling level outside the resonator zone and prevents accumulation of nitrogen in that part of the vessel where the sample is kept. Apart from this, the cotton tampon socked with liquid nitrogen does not allow defrosting of the sample.

1.2.3 EPR Spectra Recording

The EPR signal is recorded as first derivative. The line half-width was measured in mT between the points of maximum slope (Figure 1-7).