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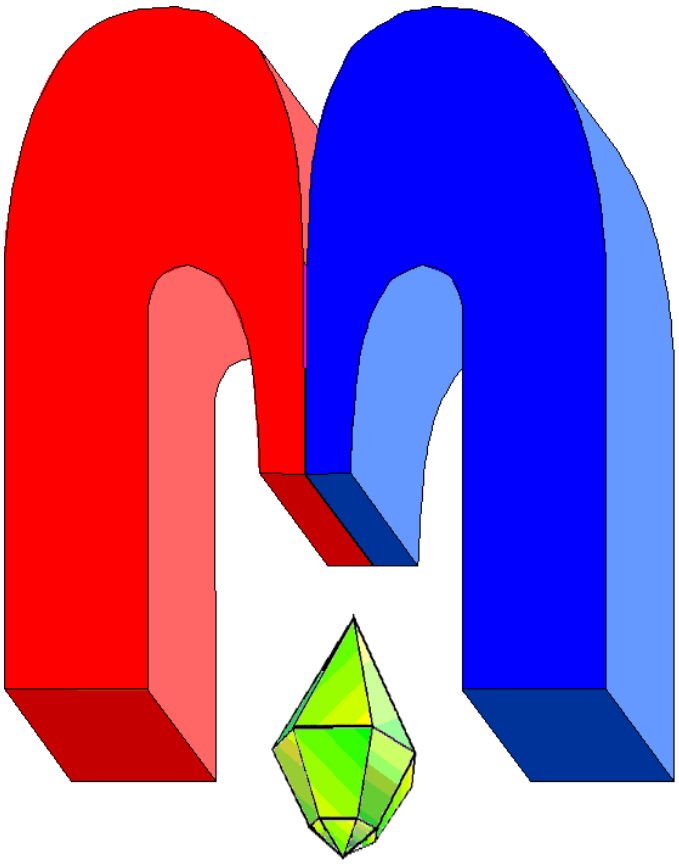

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† In Kazan University the Electron Paramagnetic Resonance (EPR) was discovered by Zavoisky E.K. in 1944.

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Identification of the signals with $g \sim 6$ in the X-band EPR spectra of human blood serum at 5-40 K†

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In the temperature range $T = 5 - 80$ K, the CW X-band EPR method was used to study blood and its serum samples taken from professional athletes, patients with connective tissue dysplasia and volunteers from the control group. At the measurement temperature of 5 K, a fairly intense absorption line with an effective g-factor of ≈ 5.84 was recorded in almost all EPR spectra of blood serum, the intensity of which decreases with increasing temperature. At temperatures above 40 K, this signal is not recorded. We believe that this absorption line arises from high-spin Fe^{3+} ions bound to the second conformation of the transferrin N-lobe iron site with $E/D \leq 0.1$. Simulation of the X-band EPR spectrum of serum recorded in the range of $500 - 2500$ G at 5 K using the EasySpin software package, in particular the "pepper" utility, allowed us to describe well all the features of the experimental spectrum from human transferrin: a double peak, a shoulder, wide wings of the signal with $g' \sim 4.3$; a weak line with $g' \sim 9.0$ and, most importantly, a signal with $g' \approx 5.84$.

PACS: 71.70.Ch, 75.10.Dg, 76.30.Kg, 71.70.Ej.

Keywords: CW EPR, X-band, blood, serum, trasferrin, ferric heme, zero field splitting, effective g-factor, helium temperatures.

1. Introduction

Iron is an important microelement necessary for the normal functioning of biological systems in the human body. The most significant function of iron ions is its participation in the binding, transportation and deposition of oxygen by hemoglobin and myoglobin. Hemoglobin is a complex protein that includes a porphyrin core containing ferrous iron. Under the influence of endogenous and exogenous factors, oxidation of heme iron from Fe^{2+} (d^6) to Fe^{3+} (d^5, high-spin ferric state) can occur, that is, the formation of methemoglobin (MetHb). Normally, the concentration of MetHb should not exceed 2% of the total hemoglobin concentration. The main carrier of iron in the body is the paramagnetic iron transport protein of blood plasma-transferrin (Tf). Each transferrin molecule can reversibly bind up to two ferric irons. This protein contains two homologous domains: the amino-terminal lobe (N-lobe) and the carboxylate-terminal lobe (C-lobe) [1]. Each lobe is divided into two subdomains between which there is a region with a distorted octahedral geometry for reversible binding of the iron ion (Fe^N or Fe^C) in the high-spin form ($S = 5/2$) [2]. One more paramagnetic plasma protein is copper-containing ceruloplasmin (Cu^{2+}-Cp). This protein, acting as ferroxidase, plays an important role in regulating the ionic state of iron, namely, it oxidizes toxic Fe^{2+} to Fe^{3+}, which is built into Tf molecules.

†This paper was selected at the International Conference devoted to the 80th anniversary of the discovery of Electron Paramagnetic Resonance "Magnetic Resonance – Current State and Future Perspectives" (EPR-80), September 23-27, 2024, Kazan, Russia. The guest Editor, Prof. M.R. Gafurov, was responsible for the publication, which was reviewed according to the standard MRSej procedure.

The presence of paramagnetic proteins in the blood has led to the widespread use of the EPR method both to obtain quantitative information on the concentration of paramagnetic ions in the blood proteins during various pathological processes in the human body and to study the structural features of these complex protein compounds. Continuous Wave (CW) X-band EPR at liquid nitrogen temperatures was predominantly used for the study blood samples (see, for example, [3]). Particular attention in the studies was paid to the analysis of the transferrin spectra with an unusual characteristic three-component signal (double peak and shoulder) near $g' \sim 4.3$, flanked by exceptionally broad tails [4-9].

From the point of view of EPR spectroscopy, transferrin is considered as a powder-like compound with a significant splitting of magnetic sublevels in zero field (ZFS). In addition, this protein is characterized by conformational inhomogeneity in the distribution of paramagnetic centers, which leads to inhomogeneous and anisotropic broadening of line, called ZFS-strain [4]. Therefore, the distribution of ZFS parameters is not described by a single value of the **D**-tensor. Principal values of **D**-tensor, as E and D, can be expressed by two parameters $D = 3/2D_z$ and $E = 1/2(D_x - D_y)$ (see, for example [4]).

A significant contribution to the numerical simulation of the characteristic double peak and shoulder in the CW X-band EPR spectra of human serum transferrin was made by M. Azarch et al. [4], who showed the need to take into account the 4^{th} order terms $B_4^{-3}\mathbf{D}_4^{-3}(\mathbf{D})$ in the spin Hamiltonian. A complete characterization of the $S = 5/2$ spin states requires the use of high-frequency EPR spectroscopy. A notable contribution to the EPR studies of human serum transferrin has been studies [4,10] using a homemade J-band CW EPR spectrometer at 275 GHz [11]. EPR studies were performed on bilobal but monoferric (Fe^N or Fe^C) human serum transferrin, which were obtained by disabling iron binding in one of the lobes through mutations. The high resolution at 275 GHz allows direct and precise determination of ZFS parameters, as well as the detection of small changes in the structure of iron binding sites. These concerns, first of all, the electronic structure of the amino-terminal lobe (N-lobe). The structure of the iron site in isolated N-lobe differs from the N-lobe site in monoferric Fe^N and in diferric Tf (contains iron ions in both binding sites) [10]. Computer simulation of J-band EPR spectra using a procedure termed the grid-of-error [4] allowed the authors to determine a significant difference in the broad distribution of ZFS parameters in both iron binding sites (Fe^N and Fe^C). In addition, the presence of a second conformation of the iron site for Fe^N-Tf was established, the electronic structure of which differs significantly from its main conformation ($E/D = 0.07$) [4,10].

Although significant advances have been made in understanding the general molecular mechanisms governing iron binding to transferrin and iron delivery to cells, questions regarding iron loading *in vivo* remain unclear not only in the norm, but in various pathologies. In particular, according to [12] Fe^{3+} ions is preferably loaded into the C-lobe iron binding site. However, this fact is in contradiction with the reported predominance of Fe^N-Tf in the serum of healthy individuals [13].

In this work, we analyze the CW X-band EPR spectra of blood serum recorded in the temperature range $T = 5 - 80$ K. At temperatures $T = 5 - 40$ K, a new single asymmetric absorption line with an effective g-factor of ≈ 5.84 was detected in almost all EPR spectra of blood serum. In this work, an attempt is made to identify this signal.

2. Materials and methods

2.1. Samples

Blood samples were collected and serum was prepared in clinical medical laboratories according to accepted standard protocols. Samples of three different groups were studied: from professional elite athletes (25 men aged 25 ± 7 years-hockey players of the Continental Hockey League); patients with connective tissue dysplasia (10 women and 5 men aged 28 – 50 years) and volunteers who obviously did not have chronic diseases (10 men aged 25 – 40 years).

Blood samples were obtained using vacutainer venous blood collection tubes. To prepare the serum samples, tubes were spun down for 10 min at 2000 g.

Within two hours after preparation of blood serum/or collection of whole blood, the same amount of sample for all measurements was placed in quartz ampoules and frozen in liquid nitrogen.

2.2. EPR spectroscopy

The EPR spectra were recorded from samples of equal volume (0.15 ml) on a Bruker EMX Plus spectrometer at a frequency of ~ 9.38 GHz with a modulation frequency of 100 KHz, modulation amplitude of 5 G, microwave power of 20 mW.

2.3. Simulation of the EPR spectra

The EPR spectra of high-spin Fe^{3+}, $S = 5/2$, were interpreted using the spin Hamiltonian, which includes fine structure terms up to the 4^{th} order [4]:

$$H = \mu_{\mathrm{B}} \mathbf{B}_0 \, \mathbf{g} \, \mathbf{S} + \mathbf{S} \, \mathbf{D} \, \mathbf{S} + \sum_q B_4^q \, \mathbf{O}_4^q \, \mathbf{S}, \tag{1}$$

where $S = 5/2$, $g = 2$ is the electron g-factor, μ_{B} is the Bohr magneton. The first term of the Hamiltonian is the Zeeman splitting in an external magnetic field. The second term describes the second-order zero-field splitting of six magnetic sublevels into three Kramers doublets. In the third term $\mathbf{O}_4^q$ is the extended Stevens operator, where q varies from -4 to $+4$.

For transferrin at X band the weak-field limit $\mu_{\mathrm{B}} \mathbf{B}_0 \, \mathbf{g} \, \mathbf{S} \ll \mathbf{SDS}$ is realized.

For simulation the X-band EPR spectra of human serum Tf, the EasySpin software package [14] was used, in particular, the "pepper" utility, designed to calculate EPR spectra from powdered solid samples. The modeling took into account the temperature dependence of the EPR signal, which made it possible to better describe the features of the experimental spectra. In the calculations, the parameters of the fine structure B_4^q the Stevens operator were taken equal to zero, except for $B_4^{-3} = 70$ MHz (by analogy with [4]).

3. Results and discussions

The relative intensities of EPR signals recorded in the spectra of human blood and serum reflect the individual characteristics of the object of study. Figure 1 shows typical EPR spectra of blood and serum recorded at 5 and 80 K. In blood serum spectrum at 80 K two absorption lines are mainly recorded. These are the signal from high-spin ($S = 5/2$) iron ions in transferrin, attributed to transitions within the $|\pm 3/2\rangle$ doublet [4,6,9] with an effective $g' \sim 4.28$, and from Cu^{2+} ions in another plasma protein, ceruloplasmin, with the main component $g_\perp$ at 2.05 [see, for example, 3]. At 80 K, the blood spectrum also shows a signal with $g \sim 5.76$, which is attributed to ferric heme in the high-spin ($S = 5/2$) state.

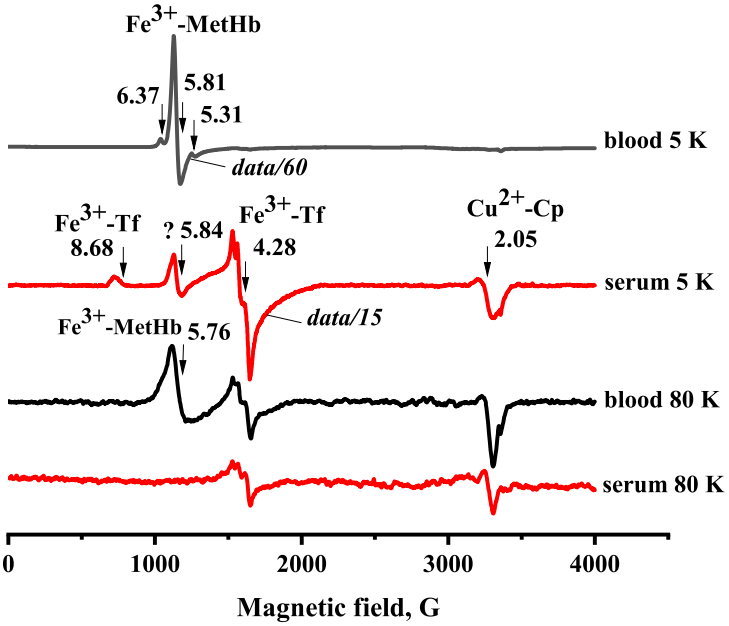

Figure 1. X-band EPR spectra of blood and serum recorded at 5 K and 80 K

At 5 K, in the spectra of serum, along with the signals from Fe^{3+}-Tf and Cu^{+2}-Cp, two more signals with $g' \sim 8.68$ and $g' \sim 5.84$ are recorded. The signal with $g' \sim 8.68$ is the second absorption line from iron ions in Tf, which is attributed to transitions within the doublets $|\pm 1/2\rangle$ and $|\pm 5/2\rangle$ [3]. The nature of the signal with $g' \sim 5.84$, detected by us in the EPR spectra of serum at helium temperatures, is the subject of research in the present work. The intensity of this new signal in the studied EPR spectra of blood serum varies quite significantly from volunteer to volunteer: from practically zero to an intensity comparable to the signal from Fe^{3+}-Tf with $g' \sim 4.28$. For $\sim 70\%$ of the serum samples studied, the signal intensity ratio (peak-to-peak) with $g' \sim 5.84$ to $g' \sim 4.28$ was $\sim 0.11 \pm 0.03$, for 25% it was slightly higher-$\sim 0.3 \pm 0.14$ and in the remaining cases it was $\sim 0.55 \pm 0.10$ (registered for athletes only).

Figure 2 shows the temperature dependence of the EPR spectra of blood. As can be seen from the figure, up to 60 K, the absorption line from high spin ferric heme ($g = 5.81$) dominates with respect to the other signals. The g-factor value obtained by us for ferric heme is close to the effective g-factor value of the high-spin form of MetHb at pH 6–8, measured at 25 K, and is equal to 5.82 [15]. The difference in intensity of the single line at $g = 5.81$ (which is the perpendicular component of the g-value in MetHb – almost of the same value along the two directions in the heme's plane) at $T \leq 10$ K and 80 K differs by almost 120 times. Moreover, in the temperature range from 60 to 80 K, a decrease in the g-factor value is observed down to 5.76 at 80 K. Along with the signal from ferric heme, two more absorption lines with g-factors of 6.37 and 5.31 are recorded in the spectra, having non-equivalence of the EPR absorption along the x and y-directions in the heme's plane. This signal is attributed to the high spin ferric heme state of erythrocyte catalase [3,16]. At temperatures above 50 K this signal is practically no longer resolved.

The temperature dependence EPR spectra of human serum are shown in Figure 3. As can be seen from figure, the intensity of the EPR signal with $g' \approx 5.84$ decreases with increasing

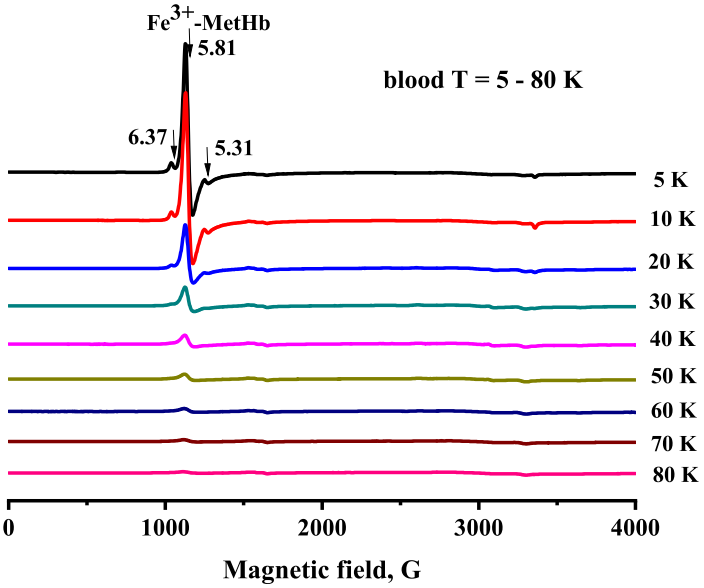

Figure 2. X-band EPR spectra of blood recorded in the temperature range from 5 to 80 K

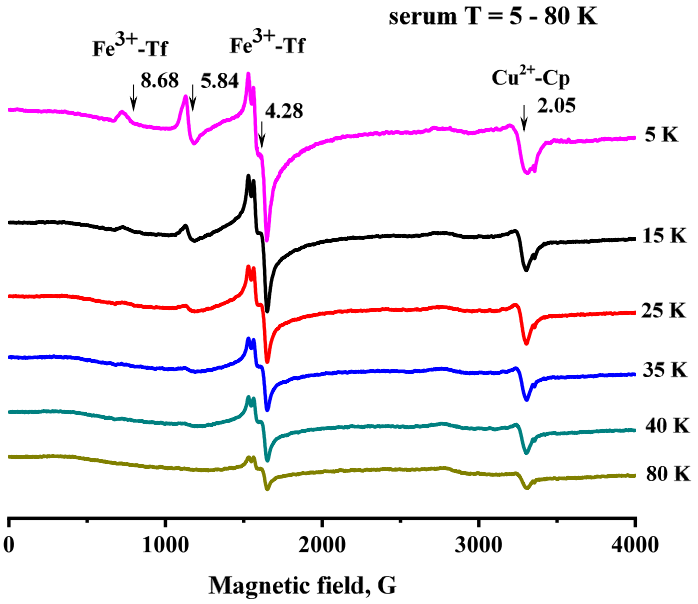

Figure 3. X-band EPR spectra of serum recorded in the temperature range from 5 to 80 K

temperature, and at temperatures above 40 K it becomes negligible. The presence of absorption lines from the high spin ferric heme state of erythrocyte catalase was not registered.

Figure 4 *a* shows the temperature dependences of the line widths from signals with $g' \approx 5.81$ in the blood spectrum and $g' \approx 5.84$ in the serum spectrum. Note that if the values of the g-factors are somewhat different, then the values of the g-factors at the maximum of the absorption line coincide and are equal to 5.94. From the data shown in the figure, it is evident that the line widths are different. Figure 4 *b* shows the temperature dependences of the normalized amplitudes of the signals from ferric heme in the blood and the signal with $g' \approx 5.84$ in the blood serum spectrum. These dependencies are described quite well by exponential functions,

with the temperature coefficients of which differ by approximately two times.

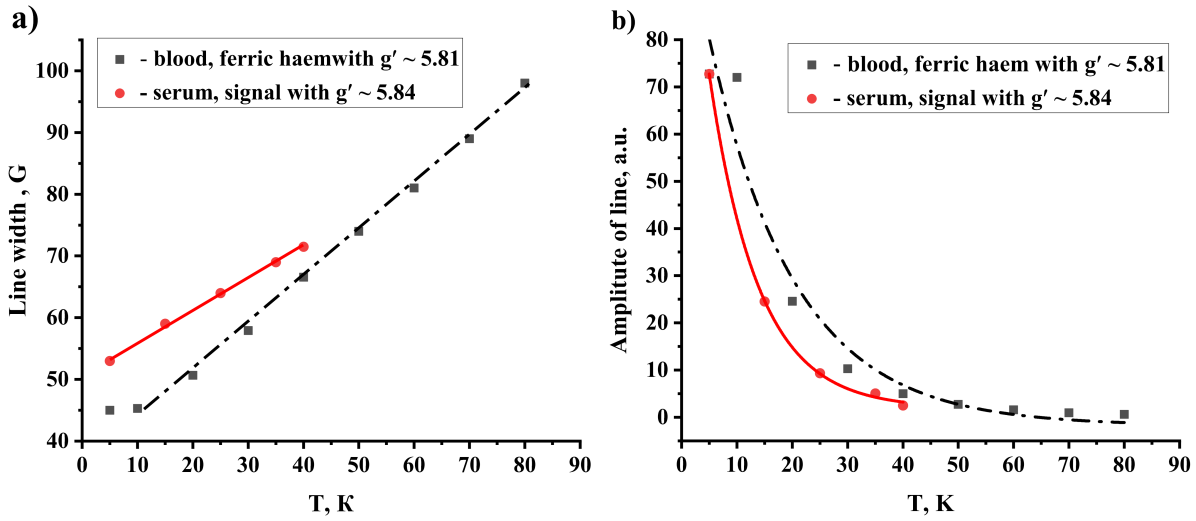

Figure 4. Temperature dependence of line width (a) and amplitude (b) for ferric heme and signal with $g' \approx 5.84$

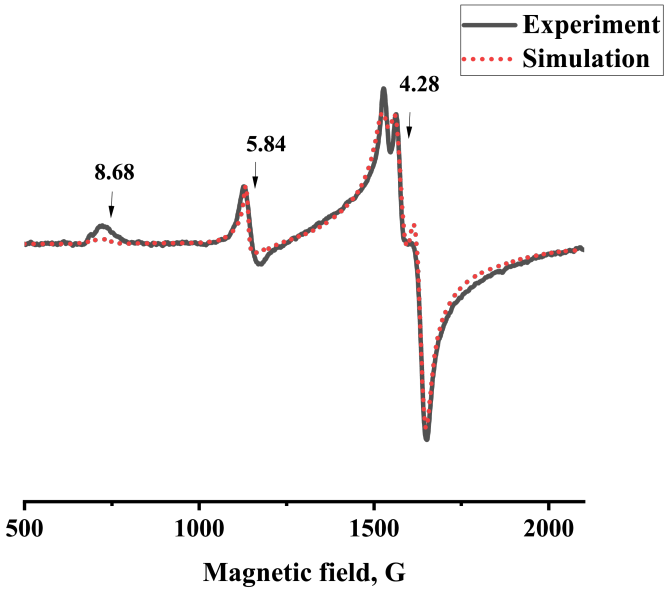

Figure 5. The part of X-band EPR spectrum (500–2500 G) of serum blood recorded at 5 K: experimental (solid line) and calculated (shot dot line) by EasySpin package

Figure 5 shows the result of simulation a part of the blood serum spectrum (magnetic field from 500 to 2500 G), recorded at 5 K. Modeling allowed us to describe quite well all the features of the experimental spectrum: a double peak, a shoulder and wide wings for the signal with $g' \approx 4.28$; a weak line with $g' \approx 8.68$ (although not very good) and, most importantly, a signal with $g' \approx 5.84$.

It is known (see, for example, [4,9]) that a signal with $g' = 4.3$ corresponds to pure rhombic symmetry ($E/D = 1/3$). From the experimental data for transferrin it follows that the effective g-factor from Fe^{3+} ions is close to 4.28, which indicates the presence of small deviations from rhombic symmetry toward axial symmetry ($E/D \leq 1/3$). Larger deviations in E/D from 1/3,

as the ligand field symmetry becomes more axial, produce the features at lower field near $g' \sim 6$ [9]. Rhombograms for $S = 5/2$, given in [4], are showing the dependence of the effective g'-value on the rhombicity ($\lambda = E/D$). The rhombograms show the possibility of the existence of the signal with $g' \sim 6.0$ attributed to transitions within the $|\pm 3/2\rangle$ doublet at $0 \leq \lambda \leq 0.1$.

It has now been established that each Tf molecule consists of two homologous domains. This suggests that they have similar, but not identical, physicochemical properties. Indeed, both theoretical calculations [6] and J-band EPR studies [4,10] have shown that the symmetry of the nearest environment of iron ions in the N and C-lobes sites is different. Deviations from rhombic symmetry toward axial symmetry for these lobes sites are also different. In addition, in the J-band spectra recorded at $T = 10\,\mathrm{K}$, the presence of a second conformation of the iron site in N-lobe with $\lambda = 0.07$ was established [4,10]. The new signal recorded by us in the X-band EPR spectrum of serum at $T < 40\,\mathrm{K}$ has a g-factor of 5.84. The rhombicity of the iron environment in this lobe site is indeed close to $\lambda < 0.1$.

As noted above, the X-band EPR studies of transferrin were usually carried out at liquid nitrogen temperature [3-5,8,9]. The exception is the works [6,7], in which measurements were performed at temperatures up to 40 K. In these studies, the signal with $g \approx 5.84$ was not detected. This is consistent with the results of our studies – this line appears at temperatures below 40 K. In the literature, we did not find data on EPR studies of blood serum at $T < 40\,\mathrm{K}$. At the same time, the authors of [4], along with the J-band EPR studies of Fe^N-Tf at helium temperatures, carried out measurements of these samples using X-band EPR at 77 K (not at temperatures below 40 K). In the experimental X-band spectrum presented in this work, the signal attributed to $\lambda \approx 0.1$ is not visible, although according to the results of their simulation it should have been there. It should be noted that in the J-band EPR spectrum from diferric Tf (containing iron ions in both binding sites) the authors [10] did not establish the presence of a signal from Fe^{3+} ions bound to the second conformation of the N-lobe site.

The question: what is the nature of the signal with $g' \sim 5.84$ detected in the EPR spectra of serum at helium temperatures? Possible options: i) the absorption line is due to the signal from iron bound to the second conformation of the transferrin N-lobe iron site (Fe^N-Tf) with $E/D \leq 0.1$, or ii) the absorption line in the spectra is the result of the presence of residual ferric heme in the studied samples, which arose during the preparation of blood serum samples in medical clinical laboratories.

Thus, the studies conducted using the X-band EPR method of blood and its serum samples showed the following. First, there is a difference in the values of g-factors: for the absorption line in serum it is equal to 5.84 ($5\,\mathrm{K} \leq T < 40\,\mathrm{K}$), while for the signal from ferric heme it is 5.81 ($5\,\mathrm{K} \leq T \leq 60\,\mathrm{K}$). Second, there are differences in the temperature dependence of line width and amplitude signals. Third, signals from the high spin ferric heme state of erythrocyte catalase were not registered in the spectra of serum. Fourth, simulation by using the EasySpin package the X-band EPR spectrum of serum recorded in the range of $500 - 2500\,\mathrm{G}$ at 5 K made it possible to describe all the features of the experimental spectrum: a double peak, a shoulder and wide wings for the signal with $g' \sim 4.28$; a weak line with $g' \sim 8.68$ (although not very good) and, most importantly, a signal with $g' \sim 5.84$.

All these factors give grounds to assume that the signal with $g' \approx 5.84$ registered in the CW X-band EPR spectra of blood serum is due to iron ions bound to human Tf. In addition, it seems most likely to us that the detected line is due to iron ions in the second conformation of the N-lobe site of transferrin.

4. Summary

In the X-band EPR spectra of blood serum, a signal with $g' \approx 5.84$ was detected, which we attribute to iron ions in transferrin. This absorption line is observed only at temperatures below 40 K. In all the studied EPR spectra of serum samples from three groups of volunteers, a signal with the indicated g-factor is clearly registered. In the temperature range from 5 to 60 K, the line from ferric heme with $g \sim 5.81$ is predominant in the corresponding EPR spectra of blood samples.

Despite all the research, the X-band EPR spectrum of transferrin and its variations have puzzled researchers for decades and remain a mystery to this day.

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