

## UNEXPECTED DETECTION OF LOW- AND HIGH-SPIN FERRIHEMOGLOBIN DERIVATIVES IN BLOOD SERUM OF POLYTRANSFUSED PATIENTS WITH HOMOZYGOUS $\beta$ -THALASSEMIA UNDER CHELATION THERAPY. AN EPR STUDY

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In a study by electron paramagnetic resonance (EPR) at 163 K on blood serum from 14 patients with homozygous  $\beta$ -thalassemia receiving transfusional and chelating therapy, a previously unnoticed low spin (LS) ferriheme resonance was detected in 8 subjects. In 7 patients weak lines with  $g$  values of  $2.24 \pm 0.04$  and  $2.16 \pm 0.02$  were seen occasionally and evidenced individual variability, and in the 8<sup>th</sup> one the corresponding resonance was intense and located at  $g = 2.28$ . Around  $g \approx 6$  methemalbumin and, in addition, other high spin (HS) ferriheme proteins were seen. After a freeze-thaw cycle, all these resonances showed  $g$  value changes, but most remarkably the  $g = 2.28$  line shifted to  $g = 2.17$ , suggesting changes in the heme environment. The  $g = 2.28$  resonance could be attributed both to a 'pure' LS state and to LS-HS dynamic spin interconversion. The newly observed species were postulated to be various methemoglobin derivatives ('acid' and 'alkaline' MetHb, and different hemichromes) released from erythrocytes. Their relations with other spectroscopic and clinical parameters are discussed. The LS and HS resonances may give insight of possible medical relevance on the incomplete suppression of patient's own erythrocyte synthesis by transfusions, on the clinical condition severity and on the electrochemical state of blood in homozygous  $\beta$ -thalassemia.

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### 1. Introduction

Applications of EPR spectroscopy [1-3] in biology, biochemistry, pharmacology and medicine developed in a dynamic, diversified and open field [4-11]. Transition ions EPR investigations of clinical interest, although not used currently for routine analysis on large numbers of patients, remain invaluable for medical research, special measurements and validation purposes. The EPR spectroscopy of blood is quasi-noninvasive and almost painless for the patient, and is experimentally simple because  $\text{Fe}^{\text{III}}$  and  $\text{Cu}^{\text{II}}$  ions are readily detected by EPR in native blood serum or plasma without preconcentration. Such studies on blood include septic shock [12],

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melanoma [13], chronic renal failure [14], ischemic heart disease [15], myocardial infarction [16],  $\beta$ -thalassemia [17, 18], and radiation-exposed workers from the Chernobyl nuclear accident [19].

In the blood serum or plasma of normal human subjects, EPR spectroscopy detects the non-heme high-spin (HS)  $\text{Fe}^{\text{III}}$ -transferrin ( $\text{Fe}^{\text{III}}$ -Tf) with its most intense line at  $g \approx 4.3$ , and the  $\text{Cu}^{\text{II}}$ -ceruloplasmin ( $\text{Cu}^{\text{II}}$ -Cp) with an easily visible line at  $g \approx 2.05$ . Their serum concentrations change in non-physiological conditions. In various diseases EPR detects new compounds of potential clinical relevance, such as methemoglobin derivatives [11 - 13]. In homozygous  $\beta$ -thalassemia, also called thalassemia major or intermedia or Cooley's anemia [20 - 24], the EPR spectra of blood serum shows in addition a specific HS signal at  $g \approx 6.0$  [17, 18, 25, 26]. This resonance is attributed to a HS  $\text{Fe}^{\text{III}}$ -methemalbumin ( $\text{Fe}^{\text{III}}$ -MHA) molecular complex with prevalent axial symmetry, absent in heterozygous  $\beta$ -thalassemia [17, 18, 25, 26]. Methemalbumin forms in serum when denatured forms of hemoglobin (methemoglobin and hemichromes, increased in thalassemia [27, 28]) are released from erythrocytes by cell membrane lysis and then it dissociates liberating heme in excess; the latter overcomes the binding capacity of the available hemopexin and thus binds to albumin [25, 26, 29].

Previously we used EPR to monitor  $\text{Fe}^{\text{III}}$ -MHA and  $\text{Fe}^{\text{III}}$ -Tf in blood serum of homozygous  $\beta$ -thalassemic children treated with transfusional therapy and deferoxamine [30]. Moreover in thalassemic serum we evidenced by simulation of the  $g \approx 6.0$  line the occurrence of a specific form of the  $\text{Fe}^{\text{III}}$ -MHA, which was spectroscopically different of the native molecular complex [31]. This distinct form of  $\text{Fe}^{\text{III}}$ -MHA showed a larger rhombic distortion of the axial crystal field around the ferric ion as compared to the ferrihemin-protein prepared *in vitro* [26]. The increased local distortion was postulated to be due to a conformational change of the albumin part of the complex and evidences the possibility of ferriproteins' alteration in the serum of the thalassemic subjects [31]. We observed also other less regular traits in the EPR spectra of the thalassemic sera, but we did not pay them sustained attention because they were only occasional and because our interest was primarily focused on the specific resonance of  $\text{Fe}^{\text{III}}$ -MHA in serum at  $g \approx 6.0$ .

Such irregular features in the EPR spectra appeared 'exotic', because they were not seen previously by this method in serum of treated thalassemic patients. Nevertheless, their incidence, even occasional and minor, may be relevant by unveiling paramagnetic compounds with unusual characteristics in the blood of certain patients and by evidencing the high interindividual variability of homozygous  $\beta$ -thalassemia. This pervasive variability in thalassemia was found by Mössbauer spectroscopy of iron proteins [32], by particle induced X-ray emission and neutron activation analysis of trace elements in serum [33], by gas-chromatographic analysis of fatty acids from erythrocytes [34] and from serum [35], and by biochemical analysis of mutations and fetal hemoglobin (HbF) levels [36].

In the present investigation we explore the potential of EPR to detect in blood serum of thalassemic patients a number of low- and high-spin ferriheme compounds occurring occasionally and detected usually by minor signals in the thalassemic serum. Our starting point was a LS line at  $g = 2.15 - 2.30$ , visible as a weak feature in the serum of some, but not all, patients, and typically observed with a rather high intensity in the case of only one subject. However we did not restrict to the high-field, LS domain of the ferriheme spectra and included the HS resonances of ferric iron around  $g \approx 6$  for two reasons. First, because in addition to the common native  $\text{Fe}^{\text{III}}$ -MHA occurring in thalassemia [17, 18, 26] we observed also its 'exotic', conformationally altered form [30] as well as a new resonance in the  $g \approx 6$  region. Second, because methemoglobin [37, 38] – present in small amounts in normal blood and elevated in certain diseases including thalassemia –, metmyoglobin [39, 40] and certain model complexes [41] show intrinsically coupled HS and LS states of the transition ion.

The EPR resonances at  $g \approx 4.3$  of hexacoordinated high-spin  $\text{Fe}^{\text{III}}$  with rhombic symmetry typical for the non-heme transferrin are not included. These lines, as well as the spectroscopic features assignable to relatively stable free radicals in serum on one hand, and certain specific responses of the EPR instrument which reveal the incidence of iron compounds with ordered magnetism on the other, will be analyzed in a subsequent article.

## 2. Theoretical background

The present study is focused mainly on EPR of Fe<sup>III</sup> in LS and HS states with resonance lines at  $g = 2.15 - 2.30$  and at  $g \approx 6$ , respectively, detected in the thalassemic blood serum. According to the  $g$  values they are typical to ferriheme proteins [42]. The LS and HS states are usually ‘pure’ and separate and belong to distinct compounds, however they may be also associated together to a mixed state [38] undergoing dynamic LS-HS spin interconversion [37-41, 43-46]. To discuss the hypothesis by which the observed lines are assigned to ferriheme proteins, – and are due either to separate pure LS or HS states or, respectively, to a mixed state undergoing dynamic LS-HS spin interconversion – we note first that even in a complex undergoing intersystem crossing, pure LS and HS states are limiting cases in a dynamic equilibrium. Therefore the necessary and sufficient background for the interpretation of all cases considered here is provided by the theories of LS and HS ferriheme iron [42, 47-51]. Note that the experimental  $g$  values exclude the rare case of intermediate spin ( $S = 3/2$ ) ferric iron which shows signals at  $g \approx 4$  and  $g \approx 2$ . The mid-spin  $S = 3/2$  state, and allowed  $5/2-3/2$  and  $1/2-3/2$  quantum mixtures including it, have only rarely been invoked to explain the spectra of certain strongly distorted porphyrins [52, 53] and hemoproteins [54].

The LS spectrum ( $S = 1/2$ ) of the low-spin  $d^5$  ferric complexes, including LS ferriheme proteins (with or without spin interconversion), with the iron ion in the multielectronic state  $^4T_1(t_2^5)$ , is described by a theory similar to that of  $d^1$  ions with an important rhombic symmetry distortion. This is the case of predominantly covalent bonds, producing a crystal field strong enough to make trivalent iron low spin. The rhombic distortion does not exceed the axial distortion of the crystal field [55]. Delocalization of  $d_{yz}$  and  $d_{xz}$  orbitals takes place in the ground state which is a combination of orbital states with  $L = 2$ , and there is incomplete orbital quenching by the ligand field [48]. Such a particular configuration explains the location of the  $g_y$  line in the 2.15 – 2.30 range (and of  $g_x$  at 1.25 – 1.93 while  $g_z$  falls at 2.40 – 3.15), resulting thus in a strongly anisotropic spectrum [51].

In our spectra the  $g_y$  line is clearly observed, while the  $g_z$  (and  $g_x$ ) lines only after smoothing. The shape and lines’ width of the EPR spectrum of the frozen serum samples are typical of a polycrystalline powder and depend on the  $g_z$ ,  $g_y$ ,  $g_x$  values and of various broadening mechanisms. As a result, the lateral low- and high-field lines are much broader as compared to the central one. For instance, 1.9:1:2.3 relative widths of the  $g_z:g_y:g_x$  lines were observed in the spectrum of cytochrome *c* peroxidase at 77 K [39]. The high-field  $g_x$  line is generally the widest even at low temperature, so much the more for our blood serum spectra recorded at 163 K. Therefore, because of broadening and noise, the  $g_x$  line may be not resolved. However, in the case of ferriheme proteins the  $g_x$  value can be calculated from the other two within the theory of the ligand field using the equation [55 – 57]:

$$|g_x| = \frac{1}{2} \left[ 4 - g_z - g_y \pm \sqrt{16 + 8(g_z + g_y) - 3(g_z^2 + g_y^2) - 2g_z g_y} \right] \quad (1)$$

Using this equation one can get the complete set of principal components of the  $g$  tensor, allowing thus a comparison with spectra of reference compounds. It gives rather accurate estimates within 1 – 2 % in many cases, however in other cases they its predictions depart by 7 – 15 % from the experimental values.

*Comparison of LS spectra.* For an overall comparison of the  $g_x$ ,  $g_y$  and  $g_z$  components in the experimental spectra and in reference compounds, the following  $g$ -fit parameter in the form of a ‘Euclidian distance’ has been introduced:

$$\delta g = \sqrt{(g_{z1} - g_{z2})^2 + (g_{y1} - g_{y2})^2 + (g_{x1} - g_{x2})^2} \quad (2)$$

Another amount which may serve for the same purpose is  $R = (g_y - g_x)$ , currently used as a measure of rhombicity of the crystal field in LS ferric complexes, and the corresponding difference is defined as a comparison parameter:

$$\delta R = |(g_{y1} - g_{x1}) - (g_{y2} - g_{x2})| \quad (3)$$

However, the relevance of  $R$  is limited and it has been shown that “decreasing  $g_z$  and increasing  $g_x$ ” would be a better criterion for increasing rhombicity [50].

Similarly, the anisotropy of the spectrum given by  $A = (g_z - g_x)$  can also be used to evaluate the fit of two spectra;  $\delta A$  has the same expression as  $\delta R$ , except that in (4) the subscript  $y$  is replaced by  $z$ . Finally, we used the sum:

$$\Sigma \delta = \delta g + \delta R + \delta A \quad (4)$$

as an empirical parameter for a synthetic evaluation of the degree of the fit between the experimental and reference LS spectra.

The HS spectrum ( $S = 5/2$ ) of the high-spin  $d^5$  ferric heme, with the iron ion in the multielectronic state  ${}^6A_1(t_2^3e^2)$ , is generally described by a spin Hamiltonian containing an isotropic Zeeman term very close to the free electron one (because  $L = 0$  for the S-state ion in the first approximation), and zero-field splitting terms with parameters  $D$  and  $E$  describing the axial and rhombic symmetry components of the crystal field, respectively, and accounting for the fine structure. In the HS ferric heme, the axial term  $D$  is unusually large ( $\sim 10 \text{ cm}^{-1}$  or more) as compared to the Zeeman splitting ( $\sim 0.3 \text{ cm}^{-1}$  for the X band), and the real  $S = 5/2$  spin system can be treated as an effective spin  $S' = 1/2$  with axial symmetry. Phenomenologically, the corresponding Zeeman interaction gives the effective  $g$  values of the experimentally observed ESR resonances at  $g_{\perp}$  and  $g_{\parallel}$ . This treatment applies to HS ferriheme compounds with weak to moderately intense crystal field, strong axial/tetragonal symmetry and predominantly ionic bonds; it works well enough for instance for MetHb. Note that this pure axial approximation requires very low values of the rhombic symmetry component  $E$  of the zero-field splitting,  $|E/D| \gtrsim 0$ , and in fact the rhombic component is completely neglected ( $E = 0$ ) [42].

For certain ferriheme proteins the complete neglect of the rhombic symmetry distortion is no longer possible and a more refined approximation is needed. For instance in the native  $\text{Fe}^{\text{III}}$ -MHA complex at 77 K the  $g_{\perp} \approx 6$  resonance is split into two resolvable lines at  $g_x = 6.089$  and  $g_y = 5.832$  [26]. The conformationally altered  $\text{Fe}^{\text{III}}$ -MHA we detected in thalassemic serum showed an even larger splitting between the  $g_x = 6.210$  and  $g_y = 5.937$  components [31]. The splitting of  $g_{\perp}$  is a measure of the departure of the heme group symmetry around the  $\text{Fe}^{\text{III}}$  ion from the tetragonal to a rhombic symmetry. To a first approximation, a distortion of this type may be expressed, according to Peisach et al [58, 59] and Kotani [49], by the ratio between the rhombic  $E$  and axial  $D$  zero-field splitting constants, which is proportional to the difference between the effective  $g_x$  and  $g_y$  values,  $E/D = (g_x - g_y)/48$ . The rhombicity may be expressed also as a percent of the total difference between a completely tetragonal and a completely rhombic field, namely  $R(\%) = 3 \cdot 100 \cdot (E/D)$ . For a less precise analysis of the spectrum, when the signal/noise ratio is not sufficiently high and the shape of the line is not extremely clear to allow the measurement of the  $g_x$  and  $g_y$  values, one finds an average position  $g_{\perp}$ :

$$g_{\perp} = g_{\perp\text{av}} = (g_x + g_y)/2 \quad (5)$$

and in the first approximation the rhombic distortion is neglected while a purely axial symmetry is assumed. In our spectra, the experimental average value  $g_{\perp} = g_{\perp\text{av}}$  describes the magnetic properties of the HS state of ferriheme iron. The  $g_{\parallel} = g_z$  line is not seen well due to broadening.

*Comparison of HS spectra.* A single  $g$  value ( $g_x$  peak maximum or  $g_{\perp\text{av}}$ ) and the line width  $\Delta B$  are the only two empirical parameters of the HS species available for identification of the serum  $g \sim 6$  resonances with spectra of reference compounds. The degree of fitness between the HS

serum spectra and the reference spectra can be evaluated by using the differences  $|\delta g| = |g_1 - g_2|$  and:

$$|\delta\Delta B|_{\text{rel}} = |\Delta B_1 - \Delta B_2|/(\Delta B_1 + \Delta B_2) \quad (6)$$

as well as the *ad hoc* defined fit parameters:

$$\Sigma\delta = |\delta g| + |\delta\Delta B|_{\text{rel}} \quad (7)$$

and:

$$[\Sigma\delta^2]^{1/2} = [(\delta g)^2 + (|\delta\Delta B|_{\text{rel}})^2]^{1/2}. \quad (8)$$

*HS-LS dynamic spin state interconversion.* Certain ferriprotein preparations such as solid powder samples of MetHb and MetMb obtained by solution evaporation with N<sub>2</sub> gas flux starting from a neutral or slightly acid pH [37, 45, 46] and lyophilized preparations [39] show inseparable LS and HS spectra. An explanation has to reconcile the two apparently incompatible situations – LS ( $S = 1/2$ ), strong field, appreciable rhombic distortion comparable to the axial symmetry component, and bonds with strong covalent character; and HS ( $S = 5/2$ ), weak to moderate field, strong axial symmetry and predominantly ionic bonds, respectively. Selection rules ( $\Delta S = 0, \pm 1$  for the spin-orbit interaction and  $\Delta S = 0$  for the electronic Zeeman interaction) forbid *quantum* mixtures, in which the wave function is a true combination of  $S = 1/2$  and  $S = 5/2$  components [54]. Therefore a HS-LS dynamic interconversion was postulated leading to equilibrium between the LS and HS states in a *thermal* mixture in the molecular ensemble.

The above mentioned authors [37, 45] found a very short ( $\sim 1$  ns) and temperature independent spin-spin relaxation time ( $T_2$ ), and a spin-lattice time ( $T_1$ ) that depends exponentially on temperature. The relaxation time  $T_2$  was of the order of the reciprocal interconversion rate between the HS and LS spin states. The dynamical thermal equilibrium was established around a critical value of the crystal field. The crystal fields with distinct symmetries are not too far away of each other, the energy levels of the multielectronic states  ${}^6A_1(t_2^3e^2)$  and  ${}^4T_1(t_2^5)$  cross for a certain intermediate field, and implicitly the two spin states fluctuate between close energies. Interconversion of LS and HS states is induced by small variations of the crystal field produced by vibrational modes together with the spin-orbit interaction and involve small displacements of the iron in and out the plane of the heme. This picture is in agreement with the spin intersystem crossing proposed on the basis of Raman spectroscopy [40, 41, 43], and its dynamical nature involving fluctuations of the protein conformation and of heme interactions with solvent water is indirectly supported by proton magnetic relaxation studies [60-62].

### 3. Experimental

#### 3.1 Human subjects

Seventeen individuals were included in the study, of which 3 were normal, healthy adults forming the control group. The remaining 14 were young patients (10 M, 4 F; age 12-25 yr; mean age  $16.5 \pm 4.0$  yr), with clinical diagnosis of severe homozygous  $\beta$ -thalassemia ambulatorily treated at Grand Hospital, Parma, Italy. They received regular erythrocyte transfusions in the range of 0.24 to 0.47 g/kg body weight/day, as well as chelation therapy with deferoxamine (Desferal<sup>®</sup>, Novartis Inc., Basel, Switzerland) in the range varying from 6.9 to 22.7 mg/kg body.day. The blood samples were taken at the end of each cycle of deferoxamine therapy ( $\sim 1$  week after the periodic injection).

#### 3.2 Blood serum samples

5 to 10 ml of blood were prelevated pretransfusionally by venipuncture for each sample. The blood samples were let to clot for 2 – 4 hours and then the sera were separated by centrifugation (1500 rpm, 10 min), subsequently recentrifugated 1 – 2 more times until they

showed no sign of coagulation and stored in the refrigerator from a few hours to a few days before recording the EPR spectra. All operations were done at 4 °C.

### 3.3 EPR spectroscopy

X-band EPR measurements were performed at 163 K using an E-line Varian spectrometer equipped with a temperature control unit and interfaced on-line with a computer. In order to prevent moisture formation the resonant cavity was flushed with nitrogen gas. Typical instrumental parameters were: modulation frequency 100 kHz, microwave frequency in the range 8.968 – 8.976 GHz, field sweep between 50 and 450 mT, microwave power 20 mW, gain  $\times 2000$ , time response 0.3 sec. Each spectrum was accumulated 16 times and subsequently processed by means of EPR3 Prometheus (Stelar, Milano) dedicated software. Volumes of 250  $\mu\text{l}$  of serum were introduced in air-tight quartz tubes of 4 mm inner diameters (Wilmad, Buena, NJ; Trimital, Milano). The absence of saturation effects was checked. Bovine ceruloplasmin in saline solution with an oxidase activity of 20-60 units/mg protein, amounting to  $\sim 100$  mg protein/mL or about  $10^{-6}$  M (Sigma-Aldrich, St. Louis, MO), partially aged, was used as a standard. The precision in  $g$  measurement of the  $g = 2.15 - 2.30$  and  $g \sim 6$  lines was better than  $\pm 0.01$ . The samples were cooled slowly to 163 K at an average rate of about  $2-4 \text{ K}\cdot\text{s}^{-1}$ .

### 3.4 Smoothing of spectra

In order to increase the visibility of the weak and broadened LS lines at  $g_z$  and  $g_x$ , the corresponding low- and high-field domains were smoothed using the Savitzky-Golay digital filter. The spectroscopic features evidenced in this way were localized by Gaussian multipeak fit and baseline subtraction. Obviously, the real lineshape of these lateral wings in the recorded derivative spectrum was rigorously different, and this empirical approach was used only as a standardized procedure for all cases. The errors in  $g_z$  and  $g_x$  values determined in this way were up to  $\pm 0.05$ , but  $g_z$  and  $g_x$  allowed comparison with the spectra of reference compounds.

### 3.5 Statistical analysis

Correlation analysis has been done in order to check the dependence of the incidence of ‘exotic’ signals in the EPR spectra on various parameters, including: incidence of other (paramagnetic) species, ‘anomalous’ magnetic properties, the hematological parameters of the subjects, their clinical condition, and the individual characteristics of therapy. The main basic data were published in the form of reprints [63] and can be obtained on request from the authors.

## 4. Results and discussions

### 4.1 Exotic LS ferriheme resonances in the blood serum from thalassemic subjects

*LS domain.* At variance to the usual trivalent iron compounds in the serum spectra of most thalassemic subjects – which show the HS  $g \approx 4.3$  line of transferrin, present also in normal blood (Fig. 1A), and in addition the HS  $g \approx 6$  signal of  $\text{Fe}^{\text{III}}$ -MHA (Fig. 1B) – minor lines with average  $g$  values of  $2.24 \pm 0.04$  and  $2.16 \pm 0.02$ , respectively, were seen occasionally in the sera of 7 patients, and one sample (no. 2) from another patient (No. 13) displayed clearly an uncommon and rather intense line located in the  $g = 2.17 - 2.28$  range (Fig. 1C and 1D).

The specific nature of these “exotic” resonances will be examined on the spectra of patient No. 13 where they show the highest relative intensity and signal-to-noise ratio. Most striking, while in the spectrum of the initially frozen serum sample this resonance was centred at  $g = 2.28$  and showed a width  $\Delta B \cong 14$  mT (Fig. 1C), the parameters of the line were changed to  $g = 2.17$  and  $\Delta B \cong 11$  mT respectively (Fig. 1D) after thawing the sample, storing at 4 °C and re-freezing it at 163 K. The resonance of transferrin ( $g \approx 4.3$ ) in these spectra, which is taken as a reference, remained essentially unchanged after the freeze-thaw cycle.

The assignment of the  $g \approx 2.28$  resonance to LS ferriheme proteins is supported strongly by spectroscopic parameters of some heme ferriproteins which show characteristic  $g_y$  values in the  $g = 2.23 - 2.28$  domain [42, 47-51, 64-67]. Examples include polycrystalline and liophylized

preparations of MetHb and MetMb [37, 39, 45, 46.] and of cytochrome c peroxidase [39] and hemichromes formed reversibly by HbA [12, 27, 68] and free  $\alpha$  chains of HbA in slightly acidic environment [57].

Similarly, the  $g = 2.17$  line also is assigned to LS ferriheme iron in proteins. [42, 47-51, 64-67]. The  $g_y$  line located in the range 2.17 – 2.21 is typical for a number of heme complexes including hydroxy form of MetHb at alkaline pH [12, 57], and hydroxy form of free  $\alpha$  chains of HbA [58]. *The drastic change of the line position after the freeze-thaw-freeze cycle from  $g = 2.28$  to  $g = 2.17$  supports the postulate that the detected low spin ferriheme is bound to a protein.* Most probably, during the freezing and thawing, the protein underwent a conformational change which influenced the environment of the heme-bound  $Fe^{III}$  ion.

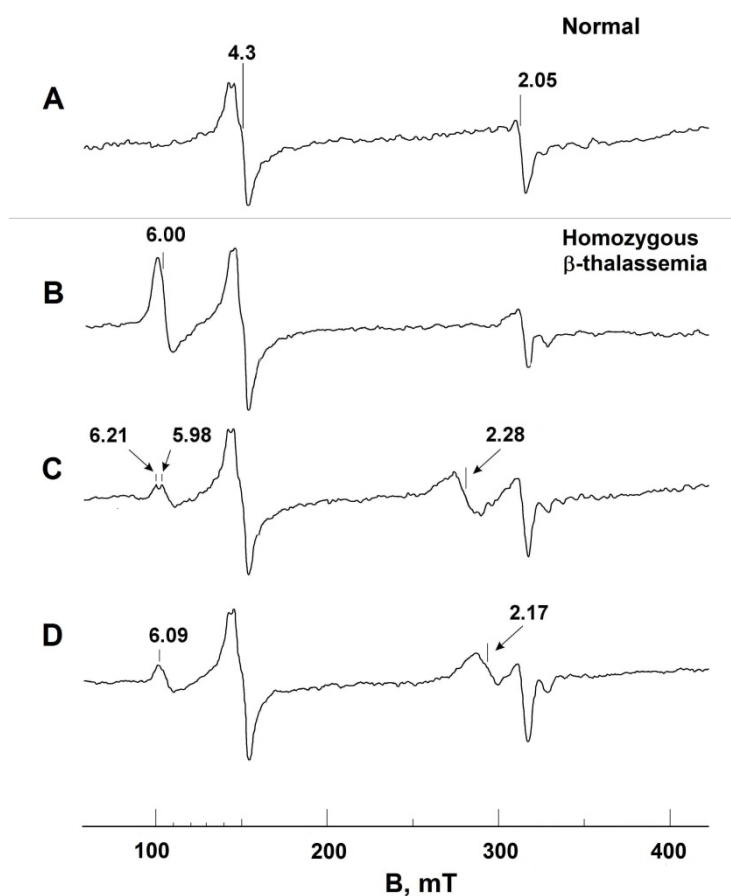


Fig. 1. EPR spectra of blood serum from normal subjects (A) and patients with homozygous  $\beta$ -thalassemia (B – D). All spectra show the  $g = 4.3$  resonance of transferrin-bound high spin ( $S = 5/2$ )  $Fe^{III}$  with rhombic symmetry and the  $g = 2.05$  line of ceruloplasmin-bound  $CuII$ . Most thalassemic sera show in addition a specific  $g = 6.0$  resonance of methemalbumin-bound high spin  $Fe^{III}$  with predominant tetragonal symmetry, and no signal in the low-spin region of the spectrum (B). A serum sample of patient 13 showed a rather intense “exotic” line at  $g = 2.28$  due to low spin ( $S = 1/2$ )  $Fe^{III}$  in a strong tetragonal crystal field with a strong rhombic distortion (C); in the high-spin region of this spectrum two overlapping resonances were detected at  $g = 6.21$  and  $g = 5.98$ . After a freeze-thaw-freeze cycle of this serum sample the low-spin resonance shifted to  $g = 2.17$  and a single line was observed in the high-spin region at  $g = 6.09$  (D). X-band EPR measurements were performed at 163 K using an E-line Varian spectrometer equipped with a temperature control unit, with the following instrumental parameters: modulation frequency 100 kHz, microwave frequency in the range 8.968 – 8.976 GHz, field sweep between 50 and 450 mT, microwave power 20 mW, gain  $\times 2000$ , time response 0.3 sec. Each spectrum was accumulated 16 times with a sweep time of 2 min.

#### 4.1 The ruling out of ferritin as a candidate for the LS spectrum

Before positively identifying the origin of the lines at  $g = 2.28$  and  $2.17$  specific to LS ferric heme, we may rule out as a candidate ferritin, the iron storage serum protein with ferroxidase activity. Only bacterial ferritin contains intrinsic heme in the coat and show LS spectra, whereas the animal ferritins, as isolated, do not [69, 70]. Degraded ferritin in the form of hemosiderin contain heme only from degradation of hemoglobin [42]. A commercial preparation of horse spleen ferritin, with an unspecified concentration but with a probable content in the range of  $30 - 100$  mg/mL as usually given by producers, showed an intense line in the  $g = 2 - 3$  region; however this line was about  $80 - 100$  mT wide [70], much wider than the lines observed in thalassemic serum. Moreover, the concentration of ferritin measured by radioimmunoassay (RIA) in the sera of the thalassemic patients, of only  $4 - 5$   $\mu\text{g/mL}$ , was too low to be detected by EPR.

Therefore the only candidates for the observed LS lines remain the ferriheme proteins; most plausibly degradation products of Hb released in serum from the erythrocytes. As MHA is known only as a HS protein complex, we look after derivatives of metHb and hemichromes.

#### 4.2 Two paradigms for the LS domain: methemoglobins and hemichromes

The  $g$  values of the two lines at  $2.28$  and  $2.17$  are consistent with several various LS hemoglobin derivatives. On one side, *aquo* and hydroxy metHb with His-Fe<sup>III</sup>-H<sub>2</sub>O and His-Fe<sup>III</sup>-OH- axial coordination are plausible candidates; on the other, hemichromes with His-Fe<sup>III</sup>-His, Cys-Fe<sup>III</sup>-His or Cys-Fe<sup>III</sup>-H<sub>2</sub>O ligation. These hemoglobin derivatives are plausible because in thalassemic patients Hb released in the blood serum may adopt these altered structures under the action of the oxidative environment. A contribution to the LS region of the spectra of Fe<sup>III</sup>-MHA is excluded because it is only in HS state both in its intact [26] and damaged form [31].

#### 4.3 The smoothed low-field domains of LS regions and possible signatures of cytochromes

A positive assignment to a certain low-spin ferric hemoglobin derivative would be possible if in addition to the  $2.28$  and  $2.17$  lines also the low-field spectroscopic feature could be observed. In our spectra recorded at  $163$  K low-field weak lines could be seen in the  $g = 2.2 - 3.5$  region, with some difficulty due to broadening and noise. Their amplitude is about one order of magnitude lower as compared to the  $g = 2.28$  and  $2.17$  lines. Therefore they could be examined only after expansion and noise reduction by smoothing (Fig. 2).

The smoothed domains of the spectrum with  $g = 2.28$  line evidenced weak signals at  $g = 2.58, 2.78, 3.08, 3.26$  and  $3.53$ , while after the freeze-thaw cycle the spectrum with  $g = 2.17$  resonance weak lines could be seen at  $2.43, 2.65, 2.88, 3.16$  and  $3.53 (\pm 0.05$  due to smoothing distortions). Of them, the lines at  $g > 3$  are usually characteristic to various cytochromes [42]. Thus the faint resonance observed at  $g \approx 3.1$  in both spectra from Fig. 1 is probably due to the low-field  $g_z = 3.03$  line of the cytochrome  $b_5$  released from erythrocytes [72, 73] where it is involved in the reduction of MetHb. Moreover,  $g_z = 3.07$  is characteristic for the mitochondrial cyt  $c$  [74] which could be released from tissue cells by membrane lysis due to lipid peroxidation oxidative radical attack. Similarly, values of  $g_z \approx 3.2$  and  $g_y \approx 2.1$  could be indicative for the cyt  $b_{558}$  from blood neutrophils [75]. Thus the minor lines located approximately at  $3.26, 3.16, 3.08$  and perhaps  $2.88$  could be tentatively assigned to cyt  $b_5$ , cyt  $c$  and cyt  $b_{558}$ , possibly involved with the oxidative stress in thalassemia: the first as a defence against Hb oxidation and the last two as markers of radical-induced cell lysis.

#### 4.4 Experiential and estimated $g$ values and postulated ferric hemoglobin derivatives in the LS spectra

Together with the central lines at  $g = 2.28$  and  $2.17$ , the weak low-field signals detected in the smoothed spectra at  $g = 2.58$  and  $2.78-2.79$  before freezing and at  $2.43$  and  $2.65-2.70$  after the freeze-thaw cycle should be consistent to the postulated ferric hemoglobin derivatives belonging to the MetHb and hemichrome classes. Using eq. (1) the unresolved high-field  $g_x$  values were estimated and the principal components ( $g_z, g_y, g_x$ ) can thus be compared to known ferriHb derivatives (Tab. 1, Fig. 2).



In the high field region, the weak line observed at  $g = 1.95$ , which may be due mainly to a  $\text{Cu}^{\text{II}}$ -serumalbumin complex [79], is the most clearly seen signal. The weak resonances corresponding to the  $g_x$  values are scantily seen in the spectra. However their  $g_x$  values calculated with eq. (1) are typical for low-spin ferriheme centres and proved to be quite informative and useful for comparison with reference compounds. Using the smoothed low-field regions for  $g_z$  and the  $g_x$  values calculated with eq. (1), each of the spectra recorded before and after the freeze-thaw cycle seem to be a superposition of two ferriHb derivative spectra (Fig. 2).

Table .1 Spectroscopic EPR parameters of serum in the LS region and of reference ferrihemoglobin derivatives

Ferriheme complex or sample	Axial ligands	$g_z^{\ddagger}$	$g_y^{\ddagger}$	$g_x$		$g_y - g_x$	$g_z - g_x$	Spin state	Ref.
				Meas. <sup>‡</sup>	Calc. <sup>*</sup>				
<i>Spectrum L1 ( Fig. 2.I, major component), before freeze-thaw cycle</i>									
Thalassemic serum (Fig. 1C)		2.78-2.79	<b>2.28</b>	-	1.64-1.65	0.63-0.64	1.13-1.15	LS or LS-HS	This work
1) MetHb-H <sub>2</sub> O polycrystalline powder	His-Fe <sup>III</sup> -H <sub>2</sub> O	2.92	<b>2.25</b>	<b>1.75*</b>	1.51	0.50	1.17	LS-HS inter-conversion	37
2) MetMb-H <sub>2</sub> O lyophilized solution	[His-Fe <sup>III</sup> -H <sub>2</sub> O]	2.95	2.28	1.70	1.43	0.58	1.25	HS-LS mixture	39
3) Cytochrome c peroxidase, dried polycrystals*		2.90	2.28	2.00	1.51	0.28	0.90	HS-LS mixture	39
		2.64	[2.28]	1.79	1.77	0.49	0.85		
4) Hb-O(salicylate)	His-Fe <sup>III</sup> -Sal.	2.95	2.26	1.47	1.46	0.79	1.48	LS	50
5) Hb-N(histidine)	His-Fe <sup>III</sup> -His	2.80	2.26	1.67	1.65	0.59	1.13	LS	50
6) Model compounds	His-Fe <sup>III</sup> -His	2.92-2.97	2.27-2.28	1.51-1.54	1.51-1.54	0.74-0.76	1.38-1.46	LS	76
7) Hemi-chrome	His-Fe <sup>III</sup> -His	2.94	2.28	1.45	1.45	0.83	1.49	LS	57
8) Hemi-chrome, H <sup>§</sup>	His-Fe <sup>III</sup> -His	2.90	2.27	1.65	1.52	0.62	1.25	LS	77
9) Hemi-chrome, (LS2) <sup>§</sup>	His-Fe <sup>III</sup> -His	2.79	<b>2.26</b>	1.65-1.66	1.66	0.60-0.61	1.13-1.14	LS	12
10) Hemi-chrome, (LS1) <sup>§</sup>	His-Fe <sup>III</sup> -His	2.72	<b>2.24</b> ,	1.73-1.75	1.73	0.49-0.51	0.97-0.99	LS	12
Ferriheme complex or sample	Axial ligands	$g_z$	$g_y$	$g_x$		$g_y - g_x$	$g_z - g_x$	Spin state.	Ref.
				Meas.	Calc.				
<i>Spectrum L2 (Fig. 2.I, minor component), before freeze-thaw cycle</i>									
Thalassemic		2.58	2.28		1.80	0.48	0.78	LS	This

serum (Fig. 1C)									work
11) Hemi-chrome, P <sup>§</sup>	Cys-Fe <sup>III</sup> -H <sub>2</sub> O	2.45	<b>2.27</b>	1.91	1.87	0.36	0.54	LS	77
12) Hemi-chrome, P <sup>§</sup>	Cys-Fe <sup>III</sup> -His	2.45	<b>2.27</b>	1.85	1.87	0.42	0.60		77
<i>Spectrum L3 (Fig. 2.II, major component), after freeze-thaw cycle</i>									
Thalassemic serum (Fig. 1D)		2.65-2.70	2.17		1.78-1.81	0.375	0.88	LS	This work
13) Hemi-chrome	His-Fe <sup>III</sup> -His	2.70	<b>2.21</b>	1.69	1.76	0.52	1.01	LS	58
14) MetHb-OH <sup>-</sup> (LS3) <sup>§</sup>	His-Fe <sup>III</sup> -OH <sup>-</sup>	2.58-2.59	<b>2.18-2.19</b>	1.83-1.84	1.84	0.35	0.75	LS	12, 57
15) HbA $\alpha$ chains- OH <sup>-</sup>	His-Fe <sup>III</sup> -OH <sup>-</sup>	2.56	<b>2.18</b>	1.88	1.86	0.30	0.68	LS	58
<i>Spectrum L4 (Fig. 2.II, minor component), after freeze-thaw cycle</i>									
Thalassemic serum (Fig. 1D)		2.43	2.17		1.91	0.26	0.52	LS	This work
16) Hemi-chrome	Cys-Fe <sup>III</sup> -His	2.40	<b>2.20</b>	1.90	1.92	0.30	0.50	LS	65, 77
17) MetHb-HO <sub>2</sub> <sup>-</sup>	His-Fe <sup>III</sup> -OOH-	2.32	2.19	1.94	1.94	0.25	0.38	LS	78
<sup>‡</sup> The errors in $g_z$ and $g_x$ values measured on smoothed spectra could be up to $\pm 0.05$ . <sup>†</sup> The precision of $g_y$ values measured directly on the original serum spectra was better than $\pm 0.01$ . <sup>*</sup> The inaccuracy of theoretical $g_x$ values estimated by eq. (1) was between $\pm 0.02$ and $\pm 0.30$ . <sup>*</sup> Two sets of $g$ values given by the authors [39]. <sup>§</sup> Notations of [12] and of [77].									

Note that the hemichromes listed in Table 1 show large variations of  $g$  values which are explained by a variety of factors. Besides the chemical nature of axial ligands (His, Cys, H<sub>2</sub>O), in dihistidine hemichromes which have the same ligands they include the (de)protonation of histidine imidazole ring [56, 12], the reciprocal orientation of the imidazole in the axial histidines [68, 76, 80, 81], and the length of the Fe-N bond with the distal histidine [12].

#### 4.5 The best fit of LS spectra with those of reference compounds

Four spectra of ferriHb derivatives detected in serum (L1 – L4), a ‘major’ and a ‘minor’ one for each of the two serum spectra before and after the freeze-thaw cycle, were recognized (Fig. 2).

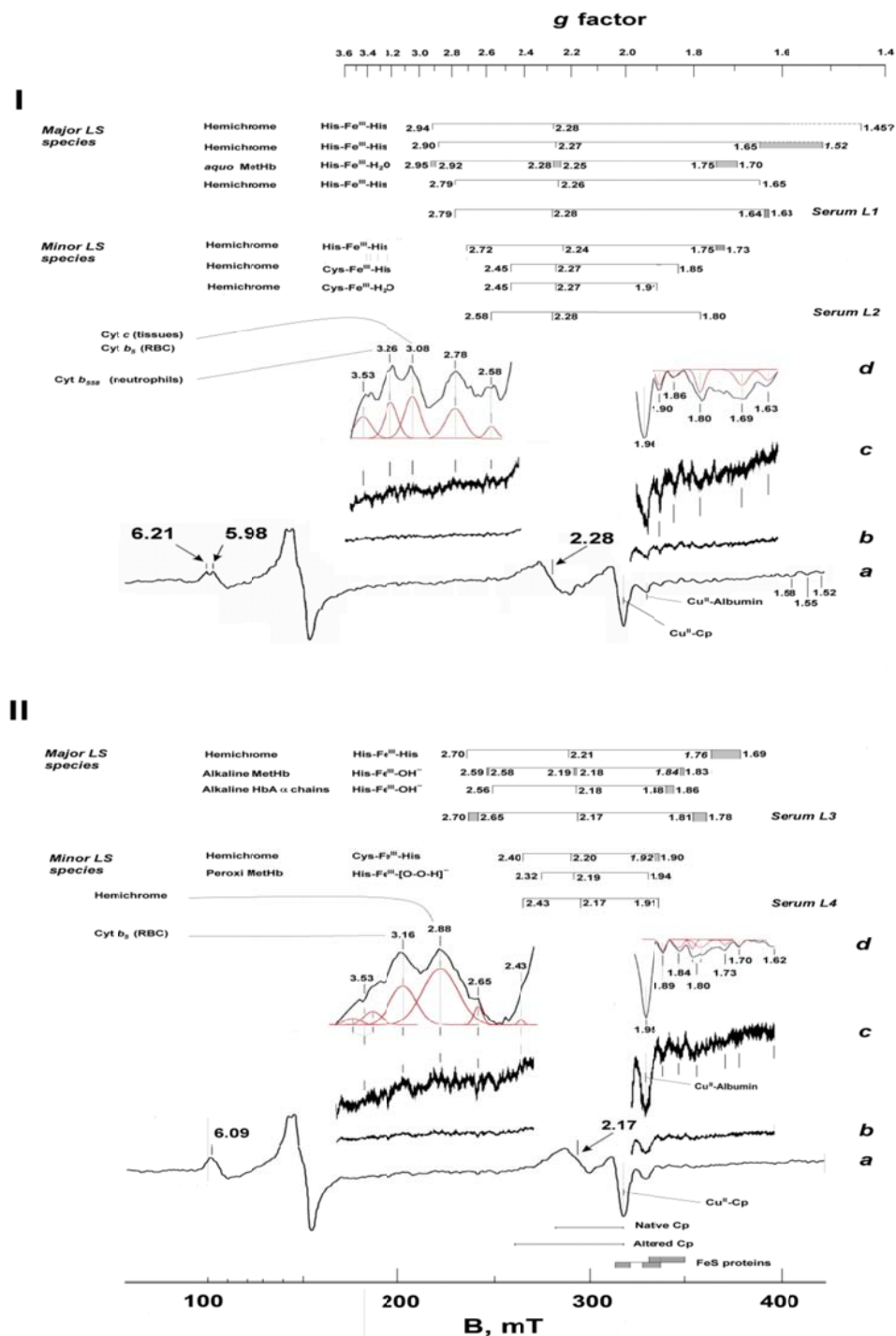


Fig. 2. Analysis of the LS region of thalassemic serum in relation with the g values of reference LS ferrihemoglobin derivatives. I, before freeze-thaw cycle, II, after cycle; a, graphically filtered spectra; b, original spectra; c, expanded x4; d, smoothed and location with Gaussians of  $g_z$  and  $g_x$  values (the baseline has been subtracted).

They are listed in Tab. 1 together with the  $g$  values of reference compounds. Table 2 presents the parameters of fit (eqs. 2-4) between these four spectra and the reference ones. Their values assist the identification by providing criteria for ordering of the plausible candidates. For instance, the reliability of the fit parameter  $\Sigma\delta$  is illustrated by the fact that it is identical up to the third decimal in the case of two different, but highly similar ferriHb derivatives, MetHb-H<sub>2</sub>O polycrystalline powder (1) and MetMb-H<sub>2</sub>O lyophilized solution (2). All discussed compounds have sets of  $g$  values which are consistent to a moderate axial symmetry and rhombic distortion [50].

*Spectrum L1, before the freeze-thaw cycle* ( $g = 2.79, 2.28, 1.64$ , Fig. 2.I). According to the parameter  $\Sigma\delta$ , the following LS compounds are assigned in order of probability to the spectrum: Hemichrome(9)[His-Fe<sup>III</sup>-His] » Hemichrome(8)[His-Fe<sup>III</sup>-His] > MetHb(1)[His-Fe<sup>III</sup>-H<sub>2</sub>O].

The  $g$  values show a high resemblance of spectrum L1 with that of dihistidine hemichrome (9) (hemichrome LS2 of [12]) and makes this compound a very probable candidate, followed by another dihistidine hemichrome (8) [76, 77] with a different spatial arrangement of the same ligands. The involvement of histidine in the sixth position, as in the postulated dihistidine hemichromes, is sustained also by the low  $\Sigma\delta$  value for the Hb-N(histidine)(5) complex [50].

Although the values of  $\Sigma\delta$  for the LS state of *aquo* metHb with His-Fe<sup>III</sup>-H<sub>2</sub>O axial coordination are a little higher, the hypothesis of ‘acid’ *aquo* metHb showing a LS-HS mixture [38] with dynamic spin state interconversion [37] still remains valid. However, the  $g$  values of spectrum L1 are somewhat different and, therefore, we have to postulate a “hemoglobin with a changed structure” with respect to HbA, as discussed below.

*Spectrum L2, before freeze-thaw cycle* ( $g = 2.58; 2.28; 1.80$ , Fig. 2.I). At the origin of this minor spectrum one of two cysteine hemichromes can be easily identified, in this order: Hemichrome(12)[Cys-Fe<sup>III</sup>-H<sub>2</sub>O] > Hemichrome(11)[Cys-Fe<sup>III</sup>-H<sub>2</sub>O]. The fit of the  $g$  values of spectrum L2 to these two similar hemichromes with Cys-Fe<sup>III</sup>-H<sub>2</sub>O axial coordination (termed P and P’ [76]) is rather modest, but there is no other candidate. The probable incidence of a Cys-Fe<sup>III</sup>-H<sub>2</sub>O hemichrome testifies for strong denaturation of metHb either in the blood.

*Spectrum L3, after freeze-thaw cycle* (2.65, 2.17, 1.81, Fig. 2.II). The set of  $g$  values of the main component after the cycle is intermediate between that of the ‘alkaline’ metHb [12, 57] and the ‘alkaline’ HbA  $\alpha$  chains-OH- [58], both with His-Fe<sup>III</sup>-OH- coordination, and a His-Fe<sup>III</sup>-His hemichrome [57]. The probability order is the following: ‘alkaline’ MetHb(14)[His-Fe<sup>III</sup>-OH<sup>-</sup>] > Hemichrome(13)[His-Fe<sup>III</sup>-His]  $\approx$  ‘alkaline’ HbA  $\alpha$  chains(15)[His-Fe<sup>III</sup>-OH<sup>-</sup>]. The ‘alkaline’ metHb is the most probable. The possible presence of the ‘alkaline’ HbA  $\alpha$  chains-OH- may imply also the incomplete synthesis suppression of endogenous erythrocytes, which contain a significant concentration of HbA  $\alpha$  chains.

*Spectrum 4, after freeze-thaw cycle* ( $g = 2.43, 2.17, 1.91$ , Fig. 2.II). The minor spectrum detected after the freeze-thaw cycle is most probably of a hemichrome with Cys-Fe<sup>III</sup>-His coordination [77]. The origin of spectrum 4 in the ‘alkaline’ hydrogen peroxide metHb-HO<sub>2</sub><sup>-</sup> [78] is less probable, but not impossible due to the increased H<sub>2</sub>O<sub>2</sub> concentration in thalassemia. Thus the order is Hemichrome(16)[Cys-Fe<sup>III</sup>-His] > ‘alkaline’ MetHb(17)[His-Fe<sup>III</sup>-HO<sub>2</sub><sup>-</sup>]. The probable incidence of a cysteine hemichrome is remarkable, because the conversion of metHb to a Cys-type hemichrome *in vitro* implies a drastic denaturation of the protein in hard conditions (e.g., heating above 60 °C) [77]. Both implications may be medically relevant by pointing to a strongly aggressive environment in the thalassemic blood.

The presence of another spectrum with  $g = 2.88, 2.17, 1.63$  after the freeze-thaw cycle may be suggested from Fig. 2, but probably it is an artifact due to smoothing and is excluded.

In brief, the completed sets of  $g$  components lead to a plausible image. Accordingly, both before and after the freeze-thaw cycle the blood serum contained probably one dominant species – either a hemichrome or a metHb with H<sub>2</sub>O or OH<sup>-</sup> in the sixth position (and, perhaps ‘alkaline’ free HbA  $\alpha$  chains, respectively) – together with a minor accompanying hemichrome. This evidences an unexpectedly complex mixture of low spin ferric hemoglobin derivatives in the blood serum of the thalassemic patient. However, the  $g$  values may be affected by smoothing-associated imprecision and have to be corroborated with other observations and aspects.

#### 4.6 Hemoglobin A and hemoglobin F as substrates of ferrihemoglobin derivatives

The assignment of the main LS spectrum found before the freeze-thaw cycle to *aquo* metHbA requires an explanation for the slightly different corresponding  $g$  values by postulating some differences in the structure of serum hemoglobin with respect to HbA. We suggest two possibilities: either a conformational alteration of HbA in the thalassemic serum, or the involvement of fetal hemoglobin (HbF) instead of HbA.

Table 2.- Parameters of fit between the EPR serum spectra and the LS reference ferrihemoglobin derivatives.

Reference compound	$\delta g$	$ \delta(g_y - g_x) $	$ \delta(g_z - g_x) $	$\Sigma\delta$
<i>Spectrum L1 (major component), before freeze-thaw cycle</i>				
MetHb-H <sub>2</sub> O polycrystalline powder (1)	0.1736	0.135	0.03	0.3386
MetMb-H <sub>2</sub> O lyophilized solution (2)	0.1739	0.055	0.11	0.3389
Hemichrome His-FeIII-His (8)	0.1155	0.015	0.11	0.2405
Hemichrome His-FeIII-His (9)	0.0229	0.03	0.005	0.0579
Hemichrome His-FeIII-His (10)	0.1219	0.135	0.16	0.4169
Hb-N(histidine) His-FeIII-His (5)	0.0354	0.045	0.01	0.0904
<i>Spectrum L2 (minor component), before freeze-thaw cycle</i>				
Hemichrome (11) Cys-FeIII-H <sub>2</sub> O	0.1706	0.12	0.24	0.5306
Hemichrome (12) Cys-FeIII-H <sub>2</sub> O	0.1396	0.06	0.18	0.3796
<i>Spectrum L3 (major component), after freeze-thaw cycle</i>				
Hemichrome His-FeIII-His (13)	0.11511	0.145	0.13	0.39011
MetHb-OH <sup>-</sup> (14)	0.09962	0.025	0.13	0.25462
HbA $\alpha$ chains-OH <sup>-</sup> (15)	0.14335	0.075	0.20	0.41835
<i>Spectrum L4 (minor component), after freeze-thaw cycle</i>				
Hemichrome Cys-Fe <sup>III</sup> -His (16)	0.0436	0.04	0.02	0.1036
MetHb-HO <sub>2</sub> <sup>-</sup> (17)	0.1158	0.01	0.14	0.2658

In the last hypothesis, the difference could be due to the presence in thalassemic serum of HbF [21], which is slightly different structurally as compared to HbA. In thalassemia, endogenous erythrocytes contain large amounts of HbF which could be released in serum if the red blood cell synthesis was not completely suppressed by transfusions. The subunit structure of HbF,  $\alpha_2\gamma_2$ , is different of the  $\alpha_2\beta_2$  structure of HbA; thus one can expect EPR-detectable differences in the iron environment. EPR has been used to study HbF but, to the best of our knowledge, only as HbNO (which is another type of system, with  $S = 1/2$  but with Fe<sup>II</sup> ion [82 – 84]) or by spin labels [85]. Therefore the precise spectroscopic EPR parameters of ferri HbF are not known. However small differences between the divalent iron environment of HbF and HbA in oxy and deoxy forms, as evidenced by the quadrupole splitting, were shown *in vitro* by Mössbauer spectroscopy [86]. Thus although we do not have a direct confirmation of HbF contribution to the LS spectrum containing the  $g = 2.28$  line, its incidence in serum with an EPR signature cannot be excluded.

Alternatively, a conformational change of the *aquo* metHbA released from transfused, exogenous aged erythrocytes could occur by structural alterations of the protein produced by chemical attack from oxygen-centered free radicals generated in thalassemic serum under the action of catalytic iron compounds.

Although the above picture of *aquo* and hidroxy metHb formed from a “serum hemoglobin with some structural differences” which could be HbF released in serum is rather speculative, it has an appeal as strong as the competing hemichrome hypothesis because it outlines the prospect of detecting by EPR the incomplete transfusional suppression of endogenous erythrocyte production; it deserves further consideration.

#### 4.7 The unusually broadened low-field lines in the LS spectra: $g$ -strain by a distribution of protein conformational alterations

In the polycrystalline powder-type LS spectra of the serum samples frozen at 163 K, the lateral lines appear to be much broader as compared to the central one. The broadening of the low-field lines at 163 K could not be attributed only to spin-lattice relaxation due to temperature. Such an effect would have led to similar changes of all lines in our spectra resulting, by analogy to the central line, in low-field lines narrower and with higher amplitude than observed. In fact this was not the case, in accordance to the fact that in the spectra of hemichromes at very low temperature the amplitude of the low-field line was much lower as compared to the central one [27]. Therefore other broadening mechanisms have to be considered.

Such broadening mechanisms should enhance appreciably the  $g_z$  line width of the ferrihemoglobin derivatives in the frozen solution above that given by the polycrystalline form function. At the same time, they should not influence significantly the  $g_y$  line, which does not show an unusual broadening. The additional mechanisms leading to a  $g$ -strain of the  $g_z$  line should not be due to heme orientation disorder, because the frozen solution is already disordered and isotropic. They could not be attributed to additional dipolar broadening due to local concentration by freezing exclusion [87], because such an effect would affect all the three lines.

Most plausibly, they could originate in certain *intramolecular* disordering with preferential effects at the level of the  $z$  axis of the heme. This may involve the axial coordinations His-Fe<sup>III</sup>-H<sub>2</sub>O and His-Fe<sup>III</sup>-OH<sup>-</sup> in the case of metHb and His-Fe<sup>III</sup>-His in the case of hemichromes. Assuming a random structural variation along the  $z$  axis of the heme (e.g., of bond length and/or angles) characterized by a distribution in the crystal field parameters of the spin Hamiltonian, this would yield a distribution in  $g_z$  values and, consequently, would lead to low-field line broadening [88, 89]. In particular, small variations in effective  $g$ -factors of the metHb in aqueous environment could be result from a distribution of distances between the oxygen of the distal water ligand and heme iron, influencing the unpaired electron density on the water molecule. A similar model was proposed for the dependence of the  $g$ -factors on distances between the distal histidine nitrogen and heme iron in hemichromes [12]. Note that the variations of the  $g_z$  and  $g_x$  extremes of the hemichromes spectra found by these authors were appreciably more important than that of the  $g_y$  central line.

The spread of water oxygen-iron or histidine-iron distances due to a random distribution of protein conformations in the heme environment could be the result of a variety of free radical-induced chemical lesions of the protein, which could undergo a diversity of molecular defects and embrace a variety of small conformational changes. Thus the later could affect either HbF or HbA suggested to be at the origin of the hemoglobin derivatives showing the  $g = 2.28$  and  $2.17$  lines.

#### 4.8 The change of LS spectrum after the freeze-thaw cycle and its molecular basis

The shift of the  $g = 2.28$  line to  $g = 2.17$  after the thermal cycle, which evidences an irreversible change dependent on freezing and thawing, favors either *aquo* metHb or a histidine hemichrome as a candidate at the origin of the initial  $g = 2.28$  resonance.

Actually, the LS spectra of metHb, metMb and other ferriheme proteins are known to show changes dependent on physical and aggregation state, on environmental conditions and on thermal treatment [46]. Thus the EPR spectra from metHb crystals “deteriorate progressively” when the protein is cycled between room and cryogenic temperatures (footnote 1 in [88]). The LS spectra and  $g$  factors of metHb and metMb derivatives and of cytochrome *c* peroxidase were different in frozen and lyophilized solution and in dried, wet and partially hydrated polycrystals [39].

The change of  $g$  value from 2.25 to 2.17 in the serum of patient No. 13 after the freeze-thaw cycle suggests an alteration in the rhombic-to-axial symmetry ratio of the crystal field and a change in the unpaired electron density on the iron ion and on the ligands. Assuming that the fifth (proximal) ligand remained unchanged, this may be due to a change of the sixth (distal) ligand's electronegativity or of the ligand-ion bond length. Such changes may be associated to changes in local electric fields [46] and/or local pressure build-up around the heme [90] during freezing.

In the hypothesis that the  $g = 2.28$  line was associated to LS *aquo* metHb with His-Fe<sup>III</sup>-H<sub>2</sub>O coordination, these mechanisms are consistent to the substitution of the H<sub>2</sub>O ligand with OH<sup>-</sup> or with a protein histidine. This would lead to the formation of the ‘alkaline’ metHb (His-Fe<sup>III</sup>-OH<sup>-</sup>) or to a hemichrome (His-Fe<sup>III</sup>-His), exhibiting the  $g = 2.17$  line. Another plausible interpretation would assume that a His-Fe<sup>III</sup>-His hemichrome yielding the initial  $g = 2.28$  line changes after the freeze-thaw to the His-Fe<sup>III</sup>-OH<sup>-</sup> ‘alkaline’ metHb. The possibility of a hemichrome-hemichrome or *aquo* metHb-*aquo* metHb transition based only on the change in the Fe<sup>III</sup>-N(His) or Fe<sup>III</sup>-OH<sub>2</sub> bond length is more unlikely. Probably only a change of relative orientations of imidazole planes could produce a relatively large change of the  $g$  factor ( $\Delta g = -0.08$ ).

Thus our LS data suggest that the  $g$  value change from 2.28 to 2.17 after the freeze-thaw cycle could be due chiefly to one of the following molecular transformations: *aquo* metHb to alkaline metHb, *aquo* MetHb to hemichrome, or hemichrome to alkaline MetHb. The thermal cycle could induce such changes *via* a conformational change of the protein, a change in the structure of the hydration layer, or both.

#### 4.9 The HS ferriheme resonances in the serum of thalassemic subjects, and their freeze-thaw cycle-induced changes

*Before the freeze-thaw cycle.* In the initial spectrum of the serum from subject No. 13, the HS line (Fig. 1-C) is split and one can distinguish two partially resolved components at  $g = 5.98$  and  $g = 6.21$ , which we postulate to represent different spectra designated as H1 and H2. Their  $g$  values and line widths  $\Delta B$  and the corresponding parameters of reference compounds spectra used for identification are presented in Table 3. Because we make use of  $\Delta B$ , the various metMb-H<sub>2</sub>O preparations studied by Yonetani and Schleyer [39] have been listed to illustrate the high variability of the HS  $g \sim 6$  line width with the different degrees of water content and crystallinity, as the later modulate the protein conformation and thus the coordination geometry around the iron ion. The degree of fitness between the HS serum and reference spectra (Table 4) are evaluated by using the differences  $|\delta g| = |g_1 - g_2|$  and  $|\delta \Delta B|_{\text{rel}}$  (eq. 6) and the fit parameters  $\Sigma \delta$  (7) and  $[\Sigma \delta^2]^{1/2}$  (8).

The first, high-field resonance at  $g = 5.98$  (spectrum H1) may be attributed to *aquo* MetHbA which binds a water molecule as the sixth (distal) ligand and has an axially symmetric EPR spectrum with  $g_x = g_y = g_z = 5.96$  [12]. Also the  $g$  value fits to *aquo* hemoglobin extracted from thalassemic blood ( $g_z = 6.0$  [17, 18]). This HS MetHb-H<sub>2</sub>O species may coexist in a pH-dependent equilibrium with a LS derivative, like a hemichrome.

Alternatively, the  $g = 5.98$  resonance in the serum of patient No. 13 may be assigned to a LS-HS dynamic interconversion of MetHb-H<sub>2</sub>O in a conformation similar to the one evidenced in solid polycrystalline powder prepared by evaporation from a 7.0 solution [37]. Although precise values of the spectroscopic parameters of the later (1 in Tab. 3) were not available for a direct comparison, similar preparations (9 and 10) of MetMb-H<sub>2</sub>O which evidenced HS-LS mixtures [39] could serve as plausible model reference compounds for HS-LS interconversion in *aquo* metHb. Depending on the hydration degree, the polycrystalline samples of *aquo* metMb showed a  $g = 6$  line 9 – 13 mT wide. The H1 line with  $\Delta B \cong 9.3$  mT linewidth matched well to the former, a result which is consistent to HS-LS spin crossover in MetHb-H<sub>2</sub>O. However, the present data are not sufficient to resolve the alternative between a LS-HS dynamic interconversion and a pure HS spin state of the *aquo* metHb; this is shown by the respective classification parameters of Tab. 4 which are very close.

Note that the position ( $g = 5.98$ ) and width ( $\Delta B \cong 9.3$  mT) of the high-field HS component show intermediate values between those of *aquo* MetHbA purified from normal erythrocytes ( $g = 5.965$ ,  $\Delta B \cong 4.0$  mT) or prepared *in vitro* at pH 7.0 (4.2 mT) [58], and from thalassemic blood ( $g_z = 6.00$ ,  $\Delta B \cong 10.0$  mT) [17]. This suggests that this line may belong to a “hemoglobin with modified structure” which may be HbA or HbF from endogenous erythrocytes.

The second, low-field component at  $g = 6.21$  (spectrum H2) may presumably come from the *aquo* form of the free  $\alpha$  chains of HbA, with  $g_x = 6.18$  [58], and from a conformationally altered form of Fe<sup>III</sup>-MHA with  $g_x = 6.210$  [31], or their mixture.

After the freeze-thaw cycle, only one line was seen at  $g = 6.09$  (spectrum H3), which fits well to the intact  $\text{Fe}^{\text{III}}$ -MHA in serum with  $g_x = 6.085$  [17, 18]. Apparently, while the initial state

Table 3. Spectroscopic EPR parameters of serum in the HS region and of HS reference ferriproteins

Ferriheme complex	Axial ligands	$g_x$	$g_y$	$g_{\perp}$		$\Delta B$ , mT	Spin state	Ref.
				Meas.	Calc.			
<i>Spectrum H1, before freeze-thawing cycle</i>								
Thalassemic serum, Spectrum 1, before cycle		5.98 (peak)				9.3	HS-LS mixture	This work
1) MetHb-H <sub>2</sub> O polycrystalline powder, evaporated from pH 7.0 solution	His-Fe <sup>III</sup> -H <sub>2</sub> O	-	-	<b>6.0</b>	-	-	LS-HS interconversion	37, 45, 46
2) MetHb-H <sub>2</sub> O monocrystal, crystallized from pH 7.0 solution	His-Fe <sup>III</sup> -H <sub>2</sub> O	-	-	<b>6.0</b>		-	HS	45, 46
3) MetHb-H <sub>2</sub> O from normal blood, frozen solution pH 7.0-7.4	His-Fe <sup>III</sup> -H <sub>2</sub> O			<b>5.8-6.0</b>		4.0	HS	57, 58
4) MetHb-H <sub>2</sub> O, from normal blood, frozen solution pH 6-9	His-Fe <sup>III</sup> -H <sub>2</sub> O	5.96–5.97 (peak)	5.82–5.83 (zero line)		5.89-5.90	4.2	HS	12
5) MetHb-H <sub>2</sub> O, from thalassemic blood, frozen solution	His-Fe <sup>III</sup> -H <sub>2</sub> O			<b>6.0</b>		10.0	HS	17, 18
6) MetMb-H <sub>2</sub> O, lyophilized solution, amorphous solid	His-Fe <sup>III</sup> -H <sub>2</sub> O			<b>6</b>		11	HS-LS mixture	39
7) MetMb-H <sub>2</sub> O, lyophilized solution, partially hydrated, amorphous solid				<b>6</b>		9		
8) MetMb-H <sub>2</sub> O, Lyophilized solution, redissolved, frozen				<b>6</b>		7		
9) MetMb-H <sub>2</sub> O, dried polycrystalline powder				<b>6</b>		13		
10) MetMb-H <sub>2</sub> O, dried polycrystalline powder, partially hydrated				<b>6±0.5</b>		9±1		
Ferriheme complex		Axial ligands	$g_x$	$g_y$	$g_{\perp}$			
				Meas.	Calc.			



<i>Spectrum H2, before freeze-thawing cycle,</i>								
Thalassemic serum, Spectrum 2, before cycle		6.21 (peak)				11.4	HS	This work
11) HbA $\alpha$ chains-H <sub>2</sub> O, frozen solution pH 7.4	His-Fe <sup>III</sup> -H <sub>2</sub> O	6.18 (peak maximum)	<b>5.78</b>		5.98	12	HS	58
12) Altered FeIII-MHA from thalassemic serum, frozen solution. Simulation	His-FeIII	6.210	5.937		6.074	7.9	HS	31
<i>Spectrum H3, after freeze-thawing cycle</i>								
Thalassemic serum, after cycle		6.09				9.7		This work
13) Native FeIII-MHA from thalassemic plasma, frozen solution	His-FeIII	6.085	5.827		5.956	8.1	HS	17, 18
14) Native FeIII-MHA prepared <i>in vitro</i> , frozen solution	His-FeIII	6.089	5.832		5.961	8.6	HS	17, 26
14) Native FeIII-MHA from thalassemic serum, frozen solution. Simulation						7.1		31

The E/D ratio which evaluate the rhombic distortion of the crystal field show three ranges of characteristic values indicative of increasing rhombicity:  $2.7\text{-}3.1 \cdot 10^{-3}$  for MetHb-H<sub>2</sub>O,  $5.3\text{-}5.6 \cdot 10^{-3}$  for Fe<sup>III</sup>-MHA, and  $\sim 8.3 \cdot 10^{-3}$  for HbA  $\alpha$  chains-H<sub>2</sub>O.

The linewidth of *aquo* MetHbA purified from normal erythrocytes (3, 4) or prepared *in vitro* (7) is smaller as compared to the protein extracted from thalassemic blood (5).

$g_{\perp}$  calculated by eq. (5).

The precision of  $g$  values measured directly on the original serum spectra was better than  $\pm 0.01$ .

of Fe<sup>III</sup>-MHA detected after the serum was freezed for the first time was somehow conformationally constrained, the respective constraint was removed or compensated after the completed freeze-thaw-freeze cycle and the intact, native protein Fe<sup>III</sup>-MHA was observed in a state corresponding to the intact, native protein complex.

In brief, the analysis of the HS ( $g = 6$ ) lines showed that before the freeze-thaw cycle the serum evidenced most probably MetHb-H<sub>2</sub>O – supposed to exist either in a pure HS state or in dynamic HS-LS interconversion equilibrium –, HbA free  $\alpha$  chains-H<sub>2</sub>O, and a conformationally altered form of Fe<sup>III</sup>-MHA; while after the cycle the native form of Fe<sup>III</sup>-MHA was the only remaining ferriheme protein in a HS state. The identified compounds are in agreement with the classification parameters of Tab. 4, which are not aimed at ranking the candidate species according to their plausibility, but to show that they are expected with comparable likelihood.

Table .4 Parameters of fit\* between the EPR serum spectra and the HS reference ferriproteins

Reference compound	$ \delta g $	$ \delta \Delta B _{\text{rel}}$	$\Sigma \delta$	$[\Sigma \delta^2]^{1/2}$
<i>Before freeze-thawing cycle, Spectrum HS1</i>				
MetMb-H <sub>2</sub> O, dried polycrystalline powder (9, 10) as model compound for MetMb-H <sub>2</sub> O with HS-LS interconversion; medie 11.0+2.8 mT	0.015	0.0773	0.0923	0.0787
MetHb-H <sub>2</sub> O, frozen solution and amorphous solid (4-8) as model for MetMb-H <sub>2</sub> O with pure HS state; medie 8.2+2.7 mT	0.015	0.0604	0.0754	0.0622
<i>Before freeze-thawing cycle, Spectrum HS2</i>				
HbA $\alpha$ chains-H <sub>2</sub> O <i>in vitro</i>	0.030	0.026	0.056	0.0397
Altered MHA from thalassemic serum	0	0.181	0.181	0.181
<i>After freeze-thawing cycle Spectrum HS3</i>				
Native MHA from thalassemic blood	0.005	0.090	0.095	0.0901
Native MHA <i>in vitro</i>	0.001	0.060	0.061	0.0600

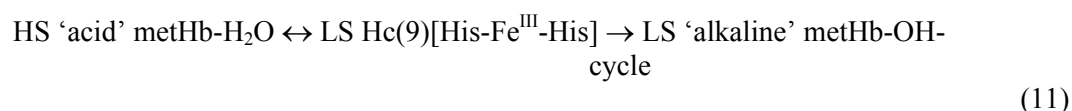
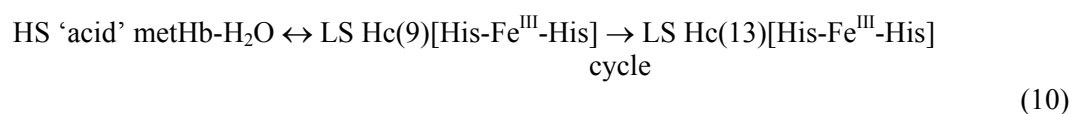
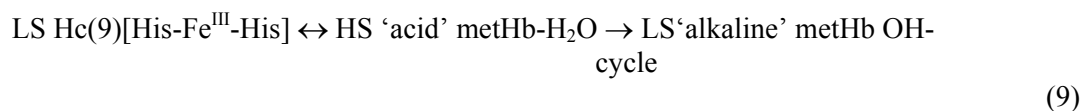
\* $|\delta g| = |g_1 - g_2|$ ;  $|\delta \Delta B|_{\text{rel}} = |\Delta B_1 - \Delta B_2| / (\Delta B_1 + \Delta B_2)$ ;  $\Sigma \delta = |\delta g| + |\delta \Delta B|_{\text{rel}}$ ;  
 $[\Sigma \delta^2]^{1/2} = [(\delta g)^2 + (|\delta \Delta B|_{\text{rel}})^2]^{1/2}$ .

#### 4.10 Physical-chemical equilibria and conversions associated to the freeze-thaw-induced changes

The effects of the freeze-thaw cycle on ferrihemoglobin derivatives are considered first, separately of those undergone by methemalbumin. One has to explain the coexistence of LS and HS ferriHb derivatives before the cycle and the incidence of only one LS species after. Because of the complexity of the serum composition and of the transformations between the initial and final states, the presence of the minor LS species in both cases is ignored and will be discussed after the major LS components.

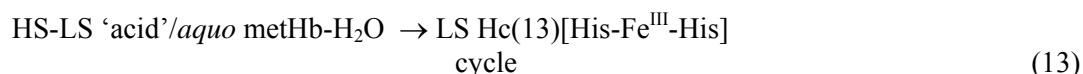
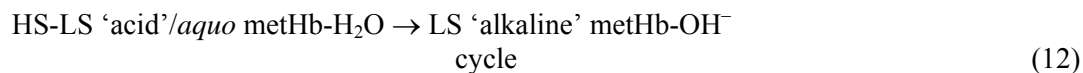
We note first that although the dihistidine hemichrome (9) fits best to the main LS spectrum detected before the cycle, it cannot be the only species present in serum because it could not explain by itself the incidence of the HS *aquo* methHb resonance. With this condition, several mechanisms are suggested.

1) Before the freeze-thaw cycle, the serum contained a mixture of the LS dihistidine hemichrome (9) with  $g = 2.28$  resonance and HS 'acid'/'*aquo*' methHb in a pH-dependent chemical equilibrium [68, 91, 12]. The cycle converts irreversibly one of these two species to a LS ferriHb derivative. Thus the equilibrium displaces on the account of the other parent compound, which decreases until both initial species disappear completely and are converted to the  $g = 2.17$  compound:

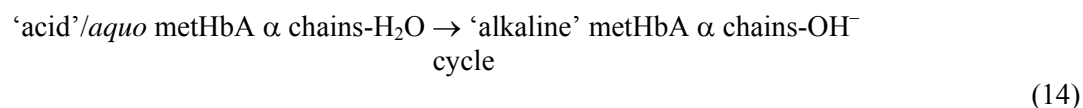


where Hc stands for hemichrome.

2) An alternative view postulates that the initial mixture of LS and HS states is due to an ‘acid’/aquo metHb-H<sub>2</sub>O derived from a “hemoglobin with a modified structure” (conformationally altered HbA or HbF) which finds itself in a temperature-driven LS-HS equilibrium with dynamic interconversion. The freeze-thaw cycle converts it to ‘alkaline’ metHb-OH<sup>-</sup> or to a hemichrome:



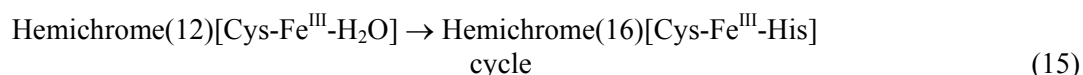
The presence of metHbA  $\alpha$  chains in serum was evidenced in the HS region of the spectra in their *aquo* or ‘acid’ form (metHbA  $\alpha$  chains-H<sub>2</sub>O) before the cycle, and in the LS region in the ‘alkaline’ form (metHbA  $\alpha$  chains-OH<sup>-</sup>) after, suggesting a reaction similar to reaction (12) involving  $\alpha$  chains instead of intact HbA ( $\alpha_2\beta_2$ ):



The mechanism of conversion should be the same in both cases. Their postulated presence in serum would imply also the incomplete suppression of endogenous erythrocyte synthesis, which in thalassemia provide free HbA  $\alpha$  chains (as well as HbF).

As a whole, the freeze-thawing of the thalassemic serum seems to favor the conversion of the major components derived from ferrihemoglobin to more basic derivatives, in a way similar to the effects which could be produced by an increase of the pH.

Finally, the minor components both before and after the freeze-thaw cycle were cysteine hemichromes; for instance:



This conversion would begin by the removal of the H<sub>2</sub>O molecule from the pocket and would conclude with the binding of the distal histidine. Both changes require the change of globin spatial structure induced by the thermal cycle.

#### 4.11 General biophysical mechanisms involved in the effects of the freeze-thaw cycle on the ferrihemoglobin derivatives

During the freeze-thaw cycle of the serum sample the prevailing apparent trend of the various Hb derivatives in either HS, LS or HS-LS states was to undergo the substitution of the ligand in the sixth position with a more electronegative group, leading generally to more basic complexes in LS states with strong rhombic distortion. The postulated substitution may result from an interplay of conformational changes of the globins, and of changes of pH, oxidoreduction potential, polarity and molecular geometry in the heme environment.

Serum pH increase during the freeze-thaw cycle may occur by conformational changes of proteins which favor intramolecular H-bond opening and binding of H<sup>+</sup> ions from the aqueous environment to previously inaccessible polar groups, as well as by selective precipitation of molecules with buffer capacity from serum [92, 93]. Such changes are known to occur in proteins, e.g. in catalase and albumin [92] and  $\beta$ -galactosidase [93], and the EPR parameters of ferrimyoglobin and cytochrome *c* peroxidase were shown to depend on preparation conditions affecting the protein hydration state [39]. Freezing-induced concentration [87] of a basic molecule from serum may also induce *via* the globins local changes of the pH.

Conformational changes of Hb produced by other factors than pH, such as binding of a small allosteric effector molecule to the protein, may induce spectroscopic effects surprisingly similar to our observations. Thus a conformational change of Hb associated to oleic acid binding changed MetHb-H<sub>2</sub>O of HS state ( $g_{\perp} = 5.8$ ), to an LS state MetHb with  $g_y = 2.19$ , almost identical to the alkaline MetHb-OH<sup>-</sup> [57].

For sure, freezing and thawing affect strongly the organization and dynamics of the bound water layer at the protein surface, which may induce changes in the spatial structure of the protein. Spin label EPR showed that surface interactions of metHb alter protein's conformation [94]. The role of hydration water in protein conformation was evidenced by EPR of HS *aquo* metHb and LS hemichromes which showed that a critical hydration providing a minimal water content covering all protein surface was necessary for reversible conformational changes [95]. Thus a conformational change due to water reorganization induced by freeze-thawing, accompanied or not by a pH change, could trigger effects leading ultimately to  $g$  values changes.

#### 4.12 The pH-dependent equilibrium and the thermal cycle-induced changes

In the case of the pH-dependent equilibrium reactions (9–11) between *aquo* metHb-H<sub>2</sub>O and dihistidine hemichrome (9) and of the cycle-induced irreversible conversion (5) of *aquo* metHb-H<sub>2</sub>O to dihistidine hemichrome (13), the exclusion or addition of a water molecule from/to the heme pocket is associated to a conformational change of the globin which brings/removes away the distal histidine in/from contact with the iron ion in the sixth position [12]. Also, in MetHb-H<sub>2</sub>O a conformational change due to water reorganization induced by freeze-thawing may expose previously buried basic groups, which thus would become able to extract H<sup>+</sup> ions from the H<sub>2</sub>O molecule bound in the sixth ligand position of the heme and convert the neutral iron-bound H<sub>2</sub>O to a ionic OH<sup>-</sup> group, as suggested by reactions (9) and (12). Reactions (9–11) presume a role for dihistidine hemichromes (8) or (10) which, depending on pH, could be involved in the equilibrium with *aquo* metHb-H<sub>2</sub>O. The hemichromes (8) – (10) differ by the anisotropy ( $g_z - g_x$ ) of their spectra, which is lower for hemichromes with parallel orientation of the imidazole ring planes and higher for those with reciprocally perpendicular orientation of the planes [76, 80]. Thus the conversion (10) of hemichrome (9) to hemichrome (13) might be due to a change in the axial histidines' reciprocal planes, possibly induced by a pH change. In any event, a significant change of (local) pH would favour any of the postulated molecular conversion mechanisms, and it is probably triggered by the freeze-thaw cycle-induced hydration layer reorganization.

Colaterally, the above discussion suggests that in future EPR studies of thalassemic serum precise measurements of the pH using microelectrodes inside the EPR quartz tubes before and after each freeze-thaw cycle might be relevant for a detailed interpretation of spectra.

#### 4.13 Further arguments for the mixed spin state equilibria in the ferrihemoglobin derivatives

The assumptions of the *aquo* metHb → alkaline metHb (reaction 12) and *aquo* MetHb → hemichrome cycle-induced conversions (reaction 13) imply that initially, i.e. before thawing and re-freezing of serum, a single and same compound was responsible for the LS resonance at  $g = 2.28$  and the HS line at  $g_{\perp} = 5.98$ . This compound could be identified as 'acid' *aquo* metHb-H<sub>2</sub>O in a mixed state undergoing dynamic LS-HS spin interconversion [37-41, 43-46]. Under the effect of the freeze-thaw cycle, the HS resonance disappeared and the LS spectrum was centred at  $g = 2.17$ . However the main  $g$  values were slightly but systematically different of those of *aquo* metHb-H<sub>2</sub>O and alkaline metHb-OH<sup>-</sup> prepared from HbA. Therefore, these species were suggested to derive from a "hemoglobin with a slightly modified structure", e.g. conformationally altered HbA or HbF from endogenous erythrocytes. Besides the  $g$  values issue, this picture is sustained by other arguments.

Various observations sustain the participation of water as a ligand in the sixth position. The LS-HS spin state interconversion was detected by EPR at low temperature in 'acid' or *aquo* MetHb with His-Fe<sup>III</sup>-H<sub>2</sub>O axial coordination prepared as a solid powder formed by evaporation of a pH 7.0 solution [37] or by lyophilization [39]. The detection of a mixture of LS and HS resonances in the spectra of metMb and their  $g$  values and linewidths were dependent on the hydration of the protein [39]. The LS-HS interconversion is favoured by the solid state of the

sample, frozen during the measurement [96], because it prevents the exchange of water with the solvent. Also low temperature may be essential because the thermal energy  $kT$  is of the order of the difference in zero-point vibrational energy between HS and LS states [90]. In fact, our frozen serum spectra were detected in the solid state. Moreover, the conversion induced by the freeze-thaw cycle evidences a high thermal treatment sensitivity, which is characteristic to metHb, metMb and other ferriheme proteins.

The LS-HS intersystem crossing shows a very high aggregation state sensitivity, as it was detected in *aquo* MetHb only in polycrystalline powder [37], while the same Hb complex in monocrystal [46] evidenced a pure HS state. This sensitivity may be due to changes in local electric fields [46] and/or local pressure build-up around the heme during freezing [90] caused by protein-induced changes as shown by resonance Raman, optical absorption, and circular dichroism spectroscopy [61]. Therefore, the successive changes of the aggregation state during the cycle could have triggered the conversion from *aquo* MetHb in LS-HS dynamic spin state equilibrium to a pure LS state compound, i.e. the alkaline MetHb or a hemichrome.

A low ratio of HS to LS concentrations was recorded at 163 K and pH  $\sim$ 7.4 in thalassemic serum (Fig. 1C) before the freeze-thaw cycle: the HS  $g = 5.98$  line is appreciably less intense than the LS  $g = 2.28$  resonance. This is expected for the LS-HS dynamic equilibrium, in agreement with a HS to LS ratio of about 0.5 at  $\sim$ 50 K in polycrystalline Hb sample prepared from pH 7.0 solution [45]. Also the high-to-low spin ratio in MetHb and MetMb was shown to depend on the cooling speed by EPR [37, 46, 97] and by optical measurements [98].

However, the situation in the liquid serum may be even more complex, if we assume that a fraction of the 'alkaline' MetHb could be in a HS state. In fact Mb at pH 11.6 was shown by Raman spectroscopy to be predominantly in the HS state although almost completely in the alkaline form, and to exhibit the existence of two kinds of HS species [99]. Nevertheless, these species were not seen by nuclear magnetic resonance (NMR) [100] and they could not be seen by EPR because the sample is frozen.

To resume, although the  $g$  values of the serum spectra did not fit perfectly to the reference spectra of *aquo* metHb and alkaline metHb, the above experimental results are consistent to the hypothesis of a "hemoglobin with a slightly modified structure" (conformationally altered HbA or HbF) in *aquo* metHb-H<sub>2</sub>O form with dynamic LS-HS spin interconversion before the freeze-thaw cycle, converted to a LS alkaline metHb or dihistidine hemichrome after (reactions 12 and 13). However, the available do not exclude the concurrent hypothesis of a complex chemical, pH-dependent equilibrium between ferriHb derivatives (reactions 9-11).

#### 4.14 The effects of the freeze-thaw cycle on the methemealbumin

Fe<sup>III</sup>-MHA is the only HS ferriprotein which retained the dominant axial symmetry of the crystal field around the Fe<sup>III</sup> ion after the freeze-thaw cycle, though with a decrease of the  $g$  factor from 6.21 to 6.09 testifying for a conformational change of albumin. These lines were assigned to the  $g_x$  components of a conformationally changed form of Fe<sup>III</sup>-MHA (6.210) and to the native protein (6.085), respectively. The  $g_y$  components could not be resolved due to noise but, presuming that they keep the known values [17, 18, 26, 31], we could estimate the ratio E/D between the rhombic and axial fine structure parameters. This ratio reduced slightly from  $5.687 \cdot 10^{-3}$  in the initial, 'altered' form of MHA to  $5.375 \cdot 10^{-3}$  in the final, 'normal' one. This indicates a slight decrease of the rhombic distortion in the environment of the Fe<sup>III</sup> ion from the first to the second freezing of the serum sample.

The situation of Fe<sup>III</sup>-MHA is different as compared to the MetHb and hemichromes, because the heme in the albumin hydrophobic pocket is almost inaccessible and the sixth ligand position remains probably unoccupied throughout the freeze-thaw cycle. Thus a conformational change of the protein may alter only the environment of the ferriheme without changing the ligand groups of the heme iron.

In its turn, the conformational change of the albumin moiety of Fe<sup>III</sup>-MHA during the freeze-thaw cycle may occur by different effects. One possible mechanism could be triggered by a phase transition of the bound water leading to the alteration of the protein spatial structure by the restructuring of its H-bond network. Another explanation for the conformationally altered Fe<sup>III</sup>-MHA could be the binding to/detachment from the albumin of a small allosteric effector present in

serum [101]. Allosteric binding may affect the symmetry around trivalent iron ion, as it happens following the non-covalent binding of fatty acids to hemoglobin [57]. Serum albumin is able to bind bilirubin, fatty acids, sterols, fat-soluble hormones, amino-acids, peptides, drugs and dyes [101-104], most of which are present in serum.

The observed variations of the  $g$  factor are explained by a plausible molecular model. We assume first that in the thalassemic serum an allosteric effector molecule was bound to the  $\text{Fe}^{\text{III}}$ -MHA and altered the protein conformation, thus yielding the  $g_x \sim 6.21$  line. This molecular configuration was dependent also on the hydration layer and was maintained in the thalassemic serum frozen initially at 163 K. After thawing at 4 °C and the subsequent freezing at 163 K the hydration layer was reorganized to a new structure which forced the allosteric effector to detach from the albumin. As a consequence, the  $\text{Fe}^{\text{III}}$ -MHA relaxed to its native conformation, characterized by the  $g_x \sim 6.09$  resonance. The model rationalizes the main experimental facts and, although it does not exclude other possible interpretations, it has the advantage of simplicity.

#### 4.15 The LS resonances in the investigated population of thalassemic subjects

In the absence of a freeze-thawing cycle the EPR spectra at 163 K of the serum samples taken from 8 of the investigated patients (subjects No. 1, 5, 6, 8, 9, 10 and 11 in addition to patient No. 13) evidenced low intensity LS resonances located in the  $g = 2.15 - 2.30$  range (Fig. 3). The shape and widths of these minor lines were similar to those shown by the serum from subject No. 13 prior and after the freeze-thawing cycle, but could not be evaluated accurately due to low intensity, noise and broadening. According to their  $g$  values, they classified together with either the one or the other of the resonances at  $g = 2.28$  and  $2.17$  of subject No. 13. Thus we found mean  $g$  values of  $2.24 \pm 0.04$  and  $2.16 \pm 0.02$ , respectively, which were significantly different of each other according to Student's  $t$  test ( $p < 0.002$ ).

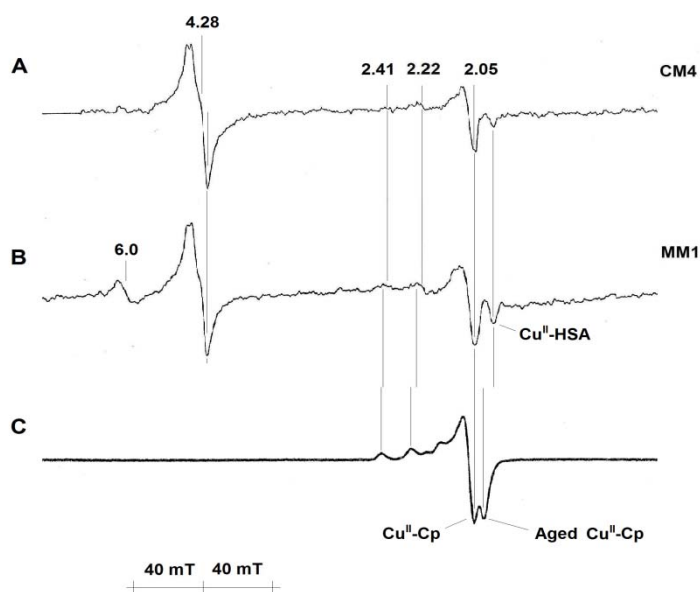


Fig. 3/2. EPR spectra showing weak low-spin resonances as observed in the blood serum from 7 patients with homozygous  $\beta$ -thalassemia of the investigated group. A single low intensity LS feature at  $g = 2.22$  is seen in a spectrum of serum from subject No. 11 (A). In this spectrum the  $g = 6$  line of  $\text{Fe}^{\text{III}}$ -MHA is practically absent, showing that the LS ferriprotein is independent of  $\text{Fe}^{\text{III}}$ -MHA. In a serum sample from patient No. 10 the weak LS line observed at the same location ( $g = 2.22$ ) is accompanied by another low intensity feature at  $g = 2.41$ , as well as by a  $g = 6.0$  resonance of significant intensity (B). The serum spectra show different spectroscopic features as compared to the spectrum of a mixture of native and partially aged  $\text{Cu}^{\text{II}}$ -Cp about  $10^{-6}$  M in saline solution (C).

In the case of two subjects who evidenced the minor LS resonances (patients No. 8 and 9), measurements of sera from the same patient taken at intervals of 3–6 months apart showed successively the two spectroscopic species, evidencing thus a possible dependence of the two LS compounds' formation on certain variations in the clinical state and serum parameters.

In a spectrum of serum from subject No. 11 a single low intensity LS feature at  $g = 2.22$  is observed (Fig. 3A). The spectrum of Fig. 3B showed also a minor line at  $g = 2.41$ . The resonance at  $g = 2.41$  is close to the component at  $g_z = 2.45$  of a hemichrome with a Cys-Fe<sup>III</sup>-His structure [77]. The line at  $g = 2.22$  is placed between the representative lines at  $g = 2.17$  and 2.28 and although many ferriHb derivatives may fit in, it is most probably a hemichrome with His-Fe<sup>III</sup>-His ( $g_y = 2.24$ ) or Cys-Fe<sup>III</sup>-His ( $g_y = 2.20$ ) axial coordination, suggesting a rather strong denaturation of hemoglobin in the thalassemic serum. The various nature of the LS compounds in different patients is not surprising, given the considerable phenotypic and genotypic heterogeneity of homozygous  $\beta$ -thalassemia [32-36].

#### 4.16 Correlation trends in the investigated population of thalassemic subjects

The occurrence of  $g = 2.15 - 2.30$  lines in sera did not show linear correlations to other parameters (e.g. free radicals, Fe<sup>III</sup>-MHA, Fe<sup>III</sup>-Tf, iron with ordered magnetism and clinical laboratory parameters) according to the generally accepted statistical significance level ( $p < 0.05$ ). This confirms that the LS ferriheme proteins are of distinct nature of these factors and relatively independent of them, as expected for ferriHb derivatives. Their occurrence only in some patients, as represented by subject No. 13 and by the other 7 patients, may represent an additional factor of individual variability in the investigated group of thalassemic patients.

Allowing for somewhat more permissive statistical requirements, certain weak linear correlation trends (e.g.  $p < 0.2$ ) of the LS lines' incidence in relation with other species and with clinical parameters appear to be relevant. Positive correlation trends with the serum level of total transferrin and Tf-bound iron shows that the  $g = 2.15 - 2.30$  resonances are associated to excess serum iron at a level where transferrin comes into play. The lack of a negative correlation trend with the level of deferoxamine therapy was consistent to the fact that the LS iron was not 'free' (decontrolled) iron but ferriheme iron, because it was not chelated by the drug. Ferriheme iron may include both compounds with LS resonances and with HS lines (including Fe<sup>III</sup>-MHA). However the LS ferriheme  $g = 2.15 - 2.30$  lines did not show any positive correlation trend with the Fe<sup>III</sup>-MHA/Fe<sup>III</sup>-Tf ratio. Also Fig. 3A, with no  $g = 6.0$  signal, shows that the minor LS lines are independent of the HS Fe<sup>III</sup>-MHA. This sustains indirectly their nature of ferriHb derivatives. Moreover these LS ferriHb derivatives evidenced a positive correlation trend with serum Hb level as expected. However, absent or slightly negative correlation trends of  $g = 2.15 - 2.30$  resonances' incidence to serum levels of unconjugated and conjugated bilirubin suggests that the LS ferriheme derivatives of hemoglobin released in serum follow another degradation pathway than the heme. In fact the "normal" heme catabolism involves mainly the ferroheme from the diamagnetic ferroHb released in the spleen by disposal of old and rigidified erythrocytes.

A special type of correlation trend is related to our previous results [30] dividing the investigated group of patients in two subgroups, characterized by different total numbers of clinical complications (A:  $2.8 \pm 1.2$ , B:  $5.3 \pm 1.1$ ). The incidence of the  $g = 2.15 - 2.30$  signals was of 50% in subgroup A and of 71% in subgroup B, respectively, suggesting that the LS resonances correlate with the most severe clinical cases. As it does not correlate clearly with separate factors which contribute to the severity of the disease (free radicals, iron with ordered magnetism, etc.), this result suggests that the occurrence of the LS signals may be related to a combination of these factors. Note that subject No. 13 with the most intense LS lines has the greatest number of clinical complications, namely 7 such syndromes.

#### 4.17 The 'exotic' character of LS ferriheme proteins in serum: physiopathological implications and Mössbauer spectroscopy of erythrocytes

Why in general, with the exception of 8 patients and especially of subject No. 13, the LS ferriHb derivatives are practically absent in the sera of other patients? Precisely, their occurrence unknown before and their comparatively high concentration only in subject No. 13 gives them an 'exotic' character. The LS ferriheme compounds derive from the *aquo* MetHb occurring in the

erythrocytes. When discharged in serum, the heme released from the Hb derivatives binds normally to hemopexin until the later is saturated; beyond this critical level it binds to albumin forming  $\text{Fe}^{\text{III}}$ -MHA. Why when released in the serum of 8 patients and mostly of subject No. 13 the ferriHb derivatives were not subsequently converted to  $\text{Fe}^{\text{III}}$ -MHA as in the case of the remaining patients? Apparently, the unusual behavior in the subgroup of 8 showed some similarity to that of patients not receiving transfusional and chelating therapy [28]. At least five factors may be taken into account – or, more probably, a combination of them:

- 1) A comparatively lower level of serum albumin, insufficient to displace the heme from the available MetHb and bind it to form  $\text{Fe}^{\text{III}}$ -MHA. The level of albumin could explain both the intense LS resonance and the relatively low intensity of the  $\text{Fe}^{\text{III}}$ -MHA  $g \approx 6$  line in the spectra of patient No. 13. Unfortunately the serum albumin values for the investigated group were not available to check this hypothesis. But serum albumin deficiency may be expected because this protein is synthesized in liver and most investigated thalassemic patients showed hepatomegaly due to iron overload.
- 2) An enhanced erythrocyte lysis releasing large amounts of ferriHb in serum. This may be due to:
  - a. An unusually high level of free radicals and other reactive oxygen species (ROS) in serum. ROS – generated in thalassemia by catalytic action of various pathologic compounds containing decontrolled iron – produce the peroxidation of the lipids from the erythrocyte membrane which thus release the hemoglobin at a high rate.
  - b. A low level of antioxidants in serum (including serum albumin), with the same enhancing effect on erythrocyte lysis.
  - c. An altered synthesis of fatty acids in the liver leading to a changed composition of the erythrocyte membrane by exchange with lipids from serum and thus to increased osmotic fragility. This may be associated to liver disfunction. Also, gas chromatographic analysis showed major alterations of fatty acid composition both in serum and in erythrocyte membrane in thalassemia [35].
- 3) An incomplete suppression of endogenous erythrocyte synthesis by transfusions. Homozygous  $\beta$ -thalassemia is primarily a hematopoietic deficiency, producing unbalanced amounts of Hb  $\alpha$  chains and high levels of HbF; the free  $\alpha$  chains oxidize and precipitate on the inner surface of the red blood cell membrane causing its lysis. This would lead also to a high concentration of HbF and of free Hb  $\alpha$  chains in serum, as already suggested by EPR results discussed before.

The last issue concerning the incomplete transfusional suppression of hematopoiesis deserves further discussion. The transfusion therapy was designed to keep the Hb level to about 12 g/L, compared to 8-9 g/L in previous years, in the attempt to inhibit as much as possible the patient's own Hb production. Therefore, most of erythrocytes of the patients were expected to come from healthy donors via transfusions. To evaluate the ratio of endogenous to exogenous red blood cells, Mössbauer studies [105] were performed in the same institute on erythrocytes prepared from blood samples taken from the same patients, including some of the samples used for the preparation of our EPR-monitored sera. Within the errors, the values of isomer shift, quadrupole shift and linewidths were comparable to those of Oxy-Hb and Deoxy-Hb from healthy subjects, suggesting that most hemoglobin was from transfused erythrocytes.

But was Mössbauer spectroscopy able to discriminate between endogenous and exogenous erythrocytes in thalassemic blood? The endogenous erythrocytes contain large amounts of HbF which could serve as a cell marker. Small differences between HbF and HbA ( $\sim 0.04$  mm/s) were evidenced in the quadrupole splitting of Oxy and Deoxy forms recorded in optimal conditions *in vitro* [86]. However Mössbauer spectroscopy is a method of limited sensitivity, and in [105] the errors were of about  $\pm 0.03$  mm/s. This would make difficult without isotopic enrichment to resolve these small differences between HbF and HbA in the noisy spectra of native erythrocytes *ex vivo*. In fact, previous Mössbauer studies did not discriminate HbA from HbF probably present in thalassemic erythrocytes [32]. Thus one may doubt whether in the quoted study [105] HbF was not



discriminated of HbA even if it was present, so that the self-produced erythrocytes could not be detected. As a consequence, the Mössbauer results [105] do not rule out the possibility that in the particular case of the patients showing LS resonances in the EPR serum spectra the transfusional suppression of patient's own hematopoiesis and endogenous Hb synthesis failed to some extent. To check this hypothesis, the search for HbF in the patients' erythrocytes is necessary. This requires further studies, e.g. by Mössbauer spectroscopy with  $^{57}\text{Fe}$  isotopic enrichment of the samples to reduce errors or by electrophoresis of extracted Hb.

#### **4.18 On the possible consequences of LS resonances for the evaluation of electrochemical parameters of blood**

The pH has been postulated above to play an essential role in the equilibrium between the LS ferriHb derivatives and in their conversions induced by the freeze-thaw cycle. More generally, another electrochemical parameter may be also involved in the 'unusual' incidence of LS compounds only in the serum of some patients. The oxidation-reduction potential is the most probable candidate in connection with the increased ROS level characteristic to thalassemia. Thus the EPR spectra might be relevant also on both pH and redox potential of serum, in addition to the incomplete inhibition of patient's own Hb and erythrocytes synthesis.

This assumption implies that the above blood parameters were different not only as compared to normal, but also in the two sub-subgroups of patients showing the LS lines, at  $g = 2.24 \pm 0.04$  and at  $g = 2.16 \pm 0.02$ , respectively. In the first case the released HS and LS compounds were apparently characteristic to neutral and slightly acidic pH including the *aquo* forms of MetHb and of free  $\alpha$  chains, as well as of hemichromes reversibly formed from HbA with a structure modified under the action of ROS, and/or from HbF. In the second, they were probably hemoglobin derivatives specific to a more alkaline environment represented by the hydroxy forms of MetHb and  $\alpha$  chains, and other hemichromes. The same argument applies to the serum samples taken from the same patient – which in successive periods showed minor LS lines located both around  $g = 2.24 \pm 0.04$  and  $g = 2.16 \pm 0.02$  (as shown by subjects No. 8 and 9). This suggests a possible relation between pH, oxidoreduction potential and ROS level both in serum and inside the erythrocytes and their influence on the EPR spectra on one hand, and the corresponding state of the organism on the other. The results provide scope for further feasibility research aimed to the development of EPR on serum iron into a new technique for probing the electrochemical state of blood from thalassemic subjects in general and for monitoring the pH in particular.

#### **4.19 On the Possible biomedical potential of the LS ferriheme detection in serum**

It is useful to resume and discuss briefly the results of potential medical interest. In the first place, the incidence of LS lines in the EPR spectrum of serum may be indicative of incomplete inhibition of patient's own Hb synthesis and high lysis of self-produced erythrocytes. Although the monitoring of transfusional therapy may be done by simple techniques at hand such as electrophoretic and thin layer chromatographic analysis of HbF, the EPR of serum may provide relevant information and insight inaccessible by these techniques.

The detection of the minor cysteine hemichromes evidences rather strong processes of Hb denaturation taking place in serum and/or erythrocytes of certain patients and absent for the others. From a medical point of view, this may reveal unusually strong oxidative stress and provides a possible criterion of differentiation within the population of thalassemic patients.

The weak line assigned to cyt  $b_5$  involved in the protection of Hb against oxidation denotes an adaptive response to an intracellular environment with strong oxidative stress inside the endogenous erythrocytes which, therefore, appears to show incomplete transfusional suppression.

The postulated detection of neutrophil cyt  $b_{558}$  suggests a possible abnormal neutrophil count in blood. Although we do not have data on the leukocyte's formula of the patients, we suppose that in many cases this disturbance was dealing with neutrophilia caused by splenectomy. In the investigated group of 14 patients, 7 were splenectomized and 4 of them (57%) showed the LS resonances of ferriHb derivatives. Also, considering the role of neutrophils as a part of the innate immune system with a role against bacterial infection, it is noteworthy that while 8 patients showed in time various infectious episodes, 4 of the remaining 6 (67%) which had no infectious complication evidenced also the LS lines. The observations suggest that cyt  $b_{558}$  is released in

serum following neutrophils' lysis due to lipid peroxidation by ROS attack. Anyway, the results point to the neutrophil count as a parameter to be monitored in further EPR studies on serum from thalassemic patients.

The weak signal attributed to mitochondrial cyt *c* could be used as marker of radical-induced lysis of the hepatic cells where this cytochrome is synthesized. Most probably, a high ROS level in the liver is associated with the insoluble iron deposits in the liver. In fact hepatomegaly, indicative of such tissular deposits, was common to 11 of the 14 investigated patients and to 7 of the 8 who showed LS lines (88%). This picture suggests that a correlation of EPR to hemochromatosis assessment by hepatic NMR tomography could be significant.

The relation between the incidence of the LS resonances in serum and hemochromatosis appeared to be indirect and complex, as the group of investigated patients was clinically heterogeneous. The results suggested that the incidence of LS resonances is favoured to some degree in the most severe cases of homozygous thalassemia. Thus EPR detection of the LS in serum may be a clinically useful hint of a possible severe form of homozygous  $\beta$ -thalassemia, possibly associated also with a combined picture of complications (e.g. an incomplete suppression of endogeneous erythrocyte synthesis, hepatomegaly, splenomegaly or splenectomy).

The identification of two sub-subgroups of patients with and without the LS lines and, within the first, of subjects showing the LS lines at  $g = 2.24 \pm 0.04$  and at  $g = 2.16 \pm 0.02$ , as well as the detection of variations of the *g* factor in successive periods in serum samples taken from the same patient confirms the inter- and intraindividual variability of homozygous thalassemia. Moreover, the incidence of the LS lines might help to discriminate between different clinical states of the patients and the present results may serve as a starting point for the development of a new technique for monitoring of the pH and electrochemical status of blood.

The EPR of serum emphasized also the interest for some particular assays currently performed in the clinical laboratory, which so far were not considered of central importance in thalassemia but which may be highly relevant (see below).

In brief, the EPR study of LS and HS ferrihemoglobin derivatives not only reveals a picture of high physiopathological and molecular complexity in a severe disorder, but also foreshadows a number of potential medical applications. The present results may lead the way for further studies developments in this direction.

#### **4.20 Potential relevance of other methods in relation with EPR of ferriheme compounds in serum**

While the present results promote EPR for further investigations on the serum of thalassemic patients aimed both at a better insight of the disease and at potential medical applications, they also suggest that increased benefits could be obtained together with other research methods and clinical laboratory techniques.

On one hand, expanding methodology – which should be done mostly using methods already employed in various studies – may include lower and variable temperature EPR with relaxation and saturation measurements [37], electron spin echo envelope modulation (ESEEM), magnetic circular dichroism (MCD) [81], Mössbauer spectroscopy [32, 81, 86, 105] however with isotopic enrichment of blood samples, UV-vis spectrophotometry [27, 28], Raman spectroscopy [38, 40, 41, 99], electrochemical measurements of pH and redox potential [106], spin trap EPR of free radicals and malonaldehyde testing of lipid peroxidation [22], and gas-chromatographic analysis of fatty acids from erythrocytes and serum [34, 35].

On the other, in addition to electrophoresis and chromatography of HbF and Hb free  $\alpha$  chains and to neutrophil count, the EPR results place in a new light current hospital laboratory techniques and paraclinical investigations. The later may include serum albumin and hepatic enzymes assay, analysis of antioxidants, evaluation of erythrocyte osmotic fragility and NMR tomography of liver.

Altogether, these methods may be of relevance, although indirectly, in order to complement the picture of trivalent iron in blood and to understand better the complex molecular pathology in thalassemia, in particular for those patients evidencing the LS ferrihemoglobin derivatives in serum. However, it is noteworthy that while methods more popular than EPR could bring some additional information, they generally will not be able to discriminate between

components of the complex mixture of Hb derivatives in serum as well as EPR, not to mention between their spin states. For instance, UV-vis spectrophotometry could detect OxiHb in addition to EPR, but in particular hardly could distinguish specifically between various histidine hemichromes differentiated only by the reciprocal orientations of their imidazole rings.

## 5. Conclusions

The occurrence of new LS resonances observed by EPR at 163 K in the blood serum of some polytransfused patients with homozygous  $\beta$ -thalassemia under chelation therapy showed an ‘exotic’ character by a number of features. Foremost, the later included its generally low intensity in 7 investigated subjects and its absence in other 6, the rarity of its manifestation as an intense  $g_y = 2.28$  ferriheme protein signal detected in only one of 50 blood serum sample taken from the group of 14 under study, and – most remarkable – its shift to  $g_y = 2.17$  after a freeze-thaw cycle. The  $g$  shifts were indicative for conformational changes of the ferriheme proteins. In the HS  $g \approx 6$  region before the thermal cycle, a conformationally altered  $\text{Fe}^{\text{III}}$ -MHA was detected together with other HS ferriheme species which appeared to coexist inseparably with the LS compounds. After the cycle, only the native  $\text{Fe}^{\text{III}}$ -MHA was observed in the HS domain.

Alltogether, the ‘exotic’ LS resonances associated with a few distinct HS lines revealed an even more complex image of the thalassemic blood serum than the usual picture, where the  $g \approx 6$  line of  $\text{Fe}^{\text{III}}$ -MHA was known to be the only specific signal in patients receiving transfusional and chelating therapy. By smoothing of spectra and theoretical estimation the  $g_z$  and  $g_x$  values were evaluated and, together with the experiential  $g_y$ , were compared to the  $g$  values of reference compounds. Thus the newly observed LS and HS signals were identified with a number of ferriHb derivatives, the most probable including the ‘acid’ *aquo* and the ‘alkaline’ hydroxide forms of MetHb, possibly involving a “hemoglobin with a modified structure” (i.e. a conformationally altered HbA, or HbF as well as free HbA  $\alpha$  chains, the later two pointing to an increased level of the fragile endogeneous erythrocytes synthesized in thalassemia), and various hemichromes. These compounds were not detected before in the thalassemic serum. Although within the available data some degree of ambiguity persists in the assignments, a dynamic picture emerged, essentially consistent with two basic possibilities for explaining the change of the LS and HS coupled signals after the freeze-thaw cycle to a single LS compound:

1) The serum contained before the freeze-thaw cycle a mixture of a LS dihistidine hemichrome with  $g = 2.28$  resonance and HS ‘acid’ *aquo* metHb in a pH-dependent chemical equilibrium. After the cycle one of these two species was irreversibly converted to a LS  $g = 2.17$  ferriHb derivative (‘alkaline’ forms of metHb and possibly free HbA  $\alpha$  chains, or dihistidine hemichrome with another reciprocal orientation of the imidazole ring planes), displacing thus the equilibrium until both initial parent species disappeared completely.

2) Alternatively, an ‘acid’ *aquo* metHb derived from a “hemoglobin with a modified structure” (conformationally altered HbA or HbF) finding itself in a temperature-driven LS-HS equilibrium with dynamic interconversion explains the initial mixture of LS and HS states, and its conversion to LS ‘alkaline’ metHb or hemichrome by the freeze-thaw cycle.

Note that based on the available data a clear-cut choice between a pH-dependent equilibrium of separate LS and HS ferriHb derivatives, or a temperature-driven LS-HS equilibrium with dynamical interconversion unfolding in only one LS species is not possible so far. At the same time, it appears that a minor cysteine-*aquo* hemichrome is converted by the thermal cycle to a related cysteine-histidine hemichrome. Its incidence suggests also strong Hb denaturation processes in blood and highlights the complexity of thalassemic serum composition.

A large variety of possible molecular mechanisms were suggested to underlie the effects of the thermal cycle. Most plausibly, the primary effect of the freeze-thawing was a conformational change of the globin proteins (methemoglobins, hemichromes and albumin) due to water reorganization in the hydration layer. The spatial structure alterations propagated in the heme environment and the EPR-detected  $g$  shift was due to ligand changes in the sixth position.

On the other hand, the conformationally altered state of Fe<sup>III</sup>-MHA – as it appeared to be when detected initially in serum – was postulated to be due to the binding by the albumin of a small molecule with allosteric modulator properties. Also, the thermal cycle dissociated probably this molecule from the protein surface, and hereby the Fe<sup>III</sup>-MHA in the native conformation resulted finally.

The biological factors postulated to be associated to the ‘exotic’ character of the LS compounds’ incidence could be a lower level of serum albumin and/or an incomplete suppression of patient’s own synthesis of erythrocytes and HbF. The endogenous red blood cells undergo lysis at a higher rate and thus favor the release in serum of a relatively high level of LS ferriheme Hb derivatives on the expense of Fe<sup>III</sup>-MHA.

The minor LS lines with average *g* values of  $2.24 \pm 0.04$  and  $2.16 \pm 0.02$  and with fluctuant occurrence in the sera of 7 patients evidenced high interindividual variability, a characteristic which is typical for thalassemia. They also switched between the two *g* values in samples taken successively from two of the subjects, showing thus various states of blood and dependence on the clinical state of the organism. The episodic incidence of low level LS ferriheme proteins may be due not only to the above biological factors, but probably also to an interplay of pH, concentrations and redox potential in the erythrocytes and serum.

The biochemical pathways of iron in the LS compounds and in the inorganic iron species, which form the object of a subsequent study now in preparation, appear to be relatively independent. Therefore the ‘exotic’ LS lines seem to be not directly relevant for a firm diagnosis of hemochromatosis. However, in the investigated group their incidence was more frequent in patients showing higher numbers of clinical complications and who were splenectomized. Thus the incidence of ferrihemoglobin derivatives in serum was more probable for patients showing more severe clinical conditions.

From a medical point of view, the detection of the ‘exotic’ LS ferriheme species in serum seems to present interest for a number of potentially relevant clinically aspects. They include mainly the possibility of monitoring the incomplete transfusional suppression of the patient’s endogenous production of erythrocytes (and the associated HbF and free HbA  $\alpha$  chains synthesis), and of evidencing a trend to develop severe clinical complications induced by iron overload. Moreover the results outline a possible new perspective for interpreting the significance of certain biochemical and biological assays accessible in the clinical laboratory (e.g., serum albumin, Hb electrophoresis, serum pH and oxidation-reduction potential), and may serve as a basis for further *ex vivo* and *in vitro* studies aimed to the development of an EPR monitoring technique of the electrochemical state of blood.

The present results grant the feasibility for ample further research directed to this purpose. However the identification of the precise nature of detected ferrihemoglobin derivatives in serum is difficult because their concentration is low in general and the incidence of representative blood samples is unpredictable. When such samples are encountered incidentally, preconcentration of serum could be necessary for extensive measurements by EPR and other spectroscopic techniques.

Notwithstanding the fact that certain aspects of our analysis are speculative to some extent, they can be tested experimentally, they promise possible practical applications, and are part of an emerging coherent picture which will necessarily have to be taken into account in order to understand better the complexity and the individual variability of homozygous  $\beta$ -thalassemia. To this purpose, the compromise between sensitivity and resolution as provided by EPR proved excellent for the analysis of ‘exotic’ hemoglobin derivatives in blood serum of patients.

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