In Vivo Hemoglobin Allotropy in Magnetic Field

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The mean magnetic moment of hem as determined in 136 blood samples from different dogs was 5.35 Bohr magneton. The individual magnetic moments varied from 4.9 to 6.7 Bohr magneton. These data are explained by the presence of hem two forms with magnetic moments of 4.9 and 6.9 Bohr magneton, respectively, in the blood. It is suggested that hem with the higher magnetic moment is able to bind two oxygen molecules with the formation of bioxyhem.

Key Words: *blood; paramagnetism; nuclear-magnetic resonance*

Equilibrium has been suggested to exist between two forms of hem: a low-spin *r*-form and a high-spin *t*-form with lower affinity to oxygen [11]. The hemoglobin saturation curve depends on *r*—*t* allosteric transition: with an increasing concentration of *r*-form the curve acquires a higher slope and shifts to the left, whereas with increasing concentration of *t*-form the curve shifts to the right having a lower slope [12]. Hem exists only in *r* form at high pH, while at low pH both forms are present [9]. Experimentally, the transition between the two forms can be observed by measuring the magnetic susceptibility of hem. Its value decreases with increasing the concentration of *r*-form and increases with increasing the concentration of *t*-form. For instance, the measurement of the magnetic susceptibility of hem crystals within the temperature range from 4.5 to 300°K by Faraday's technique showed that at 230°K magnetic moment increases from 4.9 to 5.3 Bohr magneton (μ_B) [9]. Usually, *r*—*t* transition is investigated in specially prepared samples of hemoglobin and its derivatives *in vitro*. Obviously, it is of interest to study hemoglobin allotropy *in vivo*, since it may be under control of unknown inhibitors which can be partially or completely lost during hemoglobin isolation. Therefore, in the present study a nuclear magnetic resonance (NMR) technique was applied to measure magnetic susceptibility of hemoglobin, which allowed us to make measurements in whole blood samples.

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MATERIALS AND METHODS

The measurement of hemoglobin magnetic susceptibility by *in vivo* NMR technique is based on the dependence of blood proton resonance frequency ν on the level of oxygen saturation (So_2). Hem without an added oxygen molecule has a magnetic moment $g\,\mu_B$. Hem with added oxygen becomes diamagnetic (g=0). Therefore, blood oxygenation (an increase in So_2) decreases blood magnetization (J) leading to changes in the proton resonance frequency ν. This dependence can be expressed by function:

$$\nu = A(So_2 - 1) + \nu_A, \qquad (1)$$

where ν_A is NMR frequency in saturated (arterial) blood, and the coefficient A, equal to the difference between the NMR resonance frequencies for arterial and oxygen-free blood, determines the slope of the curve **(1)**.

The A value is proportional to magnetization (J_0) induced in a blood sample by the magnetic moments of hems (g) at So_2=0:

$$A = \gamma \times \beta \times J_0, \qquad (2)$$

where γ is a proton gyromagnetic ratio and β is a coefficient defined by sample shape. For a cylindrical sample is oriented normally to the induction of the external magnetic field B, $\beta = \pi$ [10]. Magnetization J is described by Langevin formula:

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$$J_0 = \frac{\gamma^2 \times \mu^2_B \times n \times B}{3 \times k \times T}, \quad \textbf{(3)}$$

where k is Boltzman constant, T is sample temperature, and n is the number of hems in a blood volume unit.

Substitution of **(3)** into **(2)** and replacement $\gamma B = \nu$, yielded the following formula for calculation of g from the slope of curve **(1)**.

$$g = \left(\frac{3 \times A \times k \times T}{\nu \times \beta \times \mu_B^2 \times n} \right)^{1/2}. \quad \textbf{(4)}$$

Hem concentration n can be assessed considering that 1 g hemoglobin binds 1.34 ml 0_2 [6]. This assessment gives $n = 3.62 \times 10^{19} \times Hb$, where Hb is the concentration of hemoglobin (g/liter blood). Substitution of this n value and constants $\beta = \pi$, k, and μ_B into **(4)** yields dependence of g on A and n, Hb, and T values which are determined experimentally:

$$g = \left(\frac{4.3 \times 10^7 \times A \times T}{\nu \times Hb} \right)^{1/2}. \quad \textbf{(5)}$$

A high-resolution Tesla NMR spectrometer with a working frequency $\nu = 60$ MHz was used to determine A experimentally. Sample temperature was set with a thermostat. The blood proton frequency shift $\Delta\nu$ was measured in relation to the external standard hexamethyldichloroxane ($C_6H_{18}OSi_2$) at different So_2. The values of So_2 and Hb were determined using a Radiometer device. Blood samples (1 ml) were taken from artificially ventilated dogs: highly oxygenated blood was taken from arteries under conditions of oxygen respiration, low oxygenated blood — from veins under conditions of hypoxia.

RESULTS

Figure 1 illustrate the experimental dependence of the frequency shift $\Delta\nu$ on So_2. By means of the least square test this dependence can be reduced to function **(1)** with $A = 21.6 \pm 3.0$ Hz and $\nu_0 = 306 \pm 5$ Hz. Substitution of this A value and experimentally determined $n = 6 \times 10^7$ Hz, T=293°K, and Hb=156 g/liter into **(5)** yields the mean magnetic moment of hem in natural blood $g = 5.35\ \mu_B$. The obtained g value falls into the range of 5.2-5.5 μ_B previously determined by the Faraday method [11]. It agrees also with the previously reported value [6]. These data confirm the adequacy of our measuring technique.

The variability of experimental points in Fig. 1 considerably exceeds measurement errors for $\Delta\nu$ and So_2. This can be attributed to blood individual characteristics of the blood in different animals. The comparison of individual functions (Fig. 2) shows that these characteristics affect the slopes of experimental curves (coefficient A) determined by g and have no effect on ν_A. Comparing the functions obtained in dog 1 at different sample temperatures (Fig. 2) we see that heating increases the diamagnetic susceptibility (decreases ν_A) without affecting the slope.

The values of A determined from the slopes of the experimental curves (Fig. 2) are presented in Table 1. Considerable difference between the mean magnetic moments of hem in different animals can be explained in terms of equilibrium between the two allotropic forms of hemoglobin with hem magnetic moments g_1 and $g_2 > g_1$. The mean magnetic moment g in the blood depends on the relative concentration (C) of hemoglobin in form 2:

$$g = [(1-C)g_1^2 + Cg_2^2]^{1/2}. \quad \textbf{(6)}$$

With C increase from 0 to 1 the g value determined by formula **(6)** increases from g_1 to g_2. This parameter can not be below g_1 and above g_2. As seen from Table 1, g values in different dogs varied from 4.9 to 6.7 μ_B. Four data sets presented in Fig. 2 and Table 1 were selected from experimental material presented in Fig. 1 with the idea to illustrate the maximum, minimum and two intermediate g values. Therefore, 4.9 and 6.7 μ_B can be considered as the extreme possible values.

Proceeding from the spin nature of hem magnetism $g_1 = 4.9\ \mu_B$ corresponds to spin magnetic moment of 4 unpaired 3d electrons of Fe^{2+} ions, whereas $g = 6.7\ \mu_B$, the closest to $g_2 = 6.9\ \mu_B$ corresponds to the spin magnetic moment of 6 paired electrons.

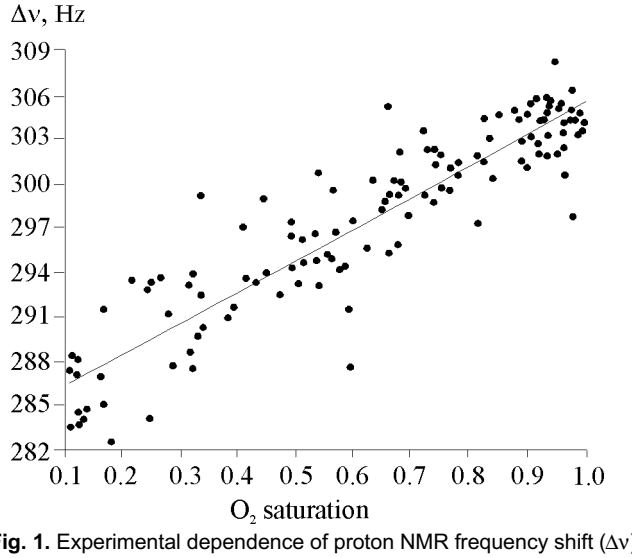

Fig. 1. Experimental dependence of proton NMR frequency shift ($\Delta\nu$) on oxygenation level in blood samples from different dogs.

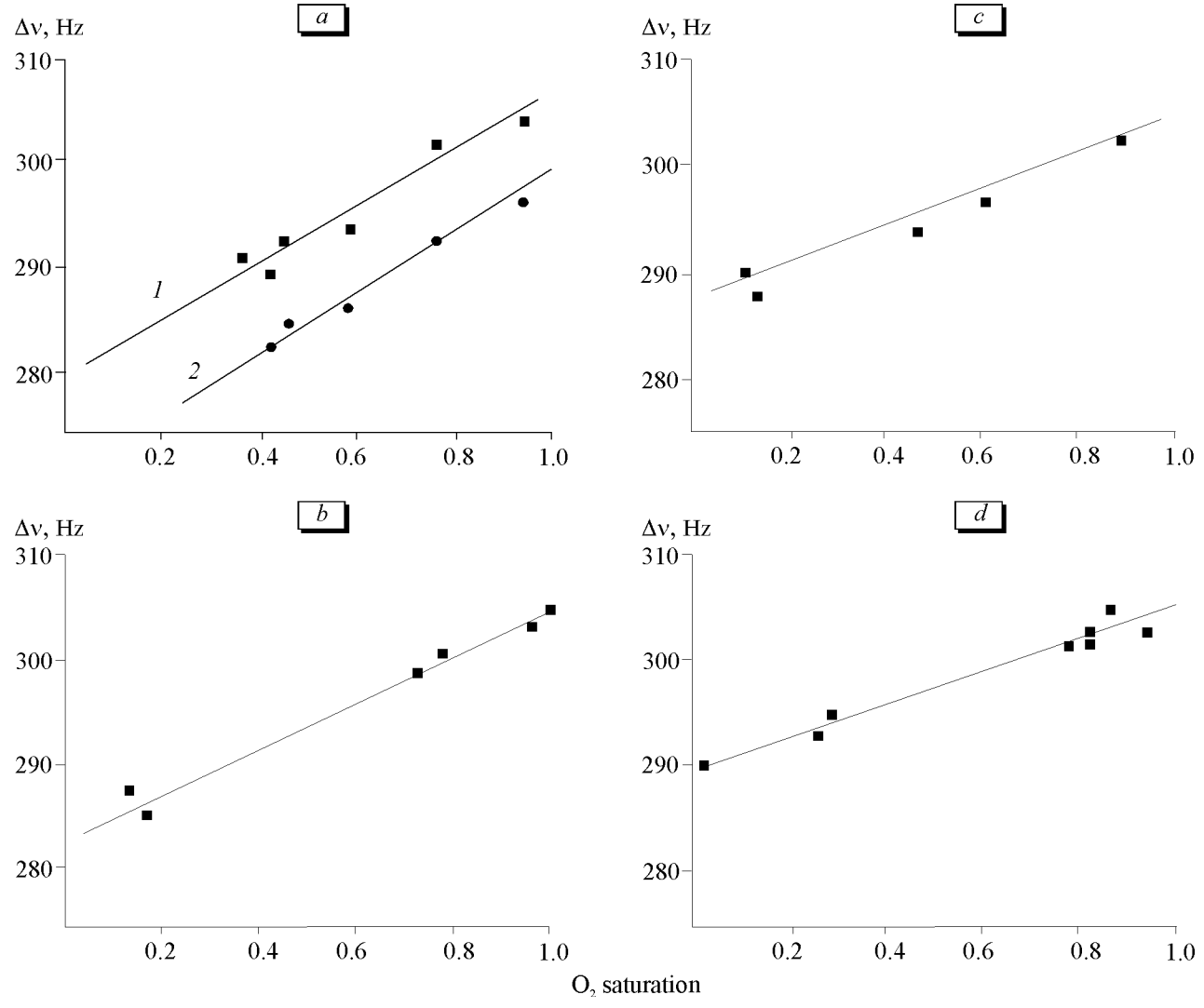

Fig. 2. Individual experimental curves reflecting dependence of proton NMR frequency shift (Δν) on blood oxygenation level in dogs. *a-d:* curves for dogs Nos. 1, 2, 3, and 4, respectively. Curves *1* and *2* for dog No. 1 were obtained at sample temperatures 25°C and 45°C, respectively.

Substituting g, g_1=4.9 μ_B and g_2=6.9 μ_B into the formula:

$$C = \frac{g^2 - g^2_1}{g^2_2 - g^2_1}, \quad (7)$$

we obtain the concentration (C) of hemoglobin in form 2 (Table 1). It can be noted that at higher pH the equilibrium is shifted to form 1, while form 2 is predominant at low pH. To confirm this correlation, the experimental data presented in Fig. 1 were redistributed in accordance with pH ranges and g values were determined within these ranges. (Table 2). The results clearly showed the existence of form 1 at high pH and of the two forms at low pH.

The affinities of form 1 and form 2 to oxygen are compared in Fig. 3 representing the dissociation curves for the same blood samples as in Fig. 2. It can be concluded, that the appearance of the dissociation curve correlates with g. The highest slope corresponds to the lowest g when hemoglobin is in form 1. With an increasing g and hemoglobin transition to form 2 the slope decreases and the curve shifts to the right. These data indicate that hemoglobin in allotropic form 2

TABLE 1. Parameters calculated from experimental curves presented in Fig. 2

Dog	1	2	3	4
A, Hz	27	23	18	16
Hb, g/liter	129	168	149	154
g	6.7	5.4	5.1	4.9
C	0.88	0.22	0.084	0
pH	7.13	7.20	7.28	7.25

TABLE 2. Experimentally determined dependence of the mean magnetic moment of hem on pH

Parameter	The number of samples				
	46	24	22	40	10
pH range	6.9-7.1	7.11-7.20	7.21-7.30	7.31-7.40	7.41-7.50
g, μ_B	5.4	5.4	5.3	5.4	4.9

possesses lower affinity to oxygen than in form 1. The analysis of the experimental results shows that hemoglobin in form 1 has a lower magnetic moment, higher affinity to oxygen and exists at higher pH, while hemoglobin in form 2 has a higher magnetic moment, lower oxygen affinity, and exists at low pH. As mentioned above, these properties are characteristic of the allosteric *r*- and *t*-forms. Therefore, it can be assumed that form 1 and 2 are the allosteric *r*- and *t*-forms, respectively, or their derivatives which are formed in the magnetic field.

We have shown previously that blood oxygen capacity increases by on average 20% after exposure to the magnetic field [4,7,8]. This effect can be explained by magnetic field-induced hem transition to allotropic *h*-form characterized by high oxygen capacity. As a rule, hem binds one oxygen molecule. An additional binding of a part of the oxygen molecule by form *h* is difficult to imagine, therefore, let us assume that hem in the *h*-form is able to bind two oxygen molecules. If each hem in this form binds additionally one oxygen molecule, the relative enhancement of the blood oxygen capacity (20%) is equal to the relative concentration of hem in form *h*. Let us compare its concentration with those of forms 1 and 2. The substitution of g_1=4.9 μ_B, g_2=6.9 μ_B, and the experimentally-determined mean value g=5.35 μ_B into expression **(7)** gives us the concentrations of hem in form 2 (C=20%) and form 1 (1-C=80%). The relative increase in the oxygen capacity turns out to be equal to the concentration of hem in form 2. Therefore, it can be assumed that the *h*-form of hem is form 2 with the magnetic moment g=6.9 μ_B.

The formation of the allotropic *h*-form in magnetic field can be caused by the attenuation of Fe^{2+} bonds with imidazole nitrogen atoms. In the allosteric *r* (relax)-form, these bonds are not tense and hence remain preserved in the magnetic field. As a result, the *r*-form manifesting itself in the magnetic field as form 1 retains its usual oxygen capacity. In the allotropic *t* (tense)-form, these bonds are tense and become deteriorated after attenuation by the magnetic field. As a result, hem undergoes transition from *t* to *h*-form, capable to bind an additional oxygen molecule instead of imidazole.

An increased blood oxygen capacity is maintained for several minutes after magnetic field withdrawal [8], suggesting the *h*-form stability. Exposure to the magnetic field increases pH [3] indicating that hem transition to form *h* increases its basic properties. Different concentrations (C) of form *h* in Table 1 imply that $r \rightarrow t$ or $t \rightarrow h$ transitions involve some inhibitors whose activity or concentration strongly depend on the individual characteristics of the organism.

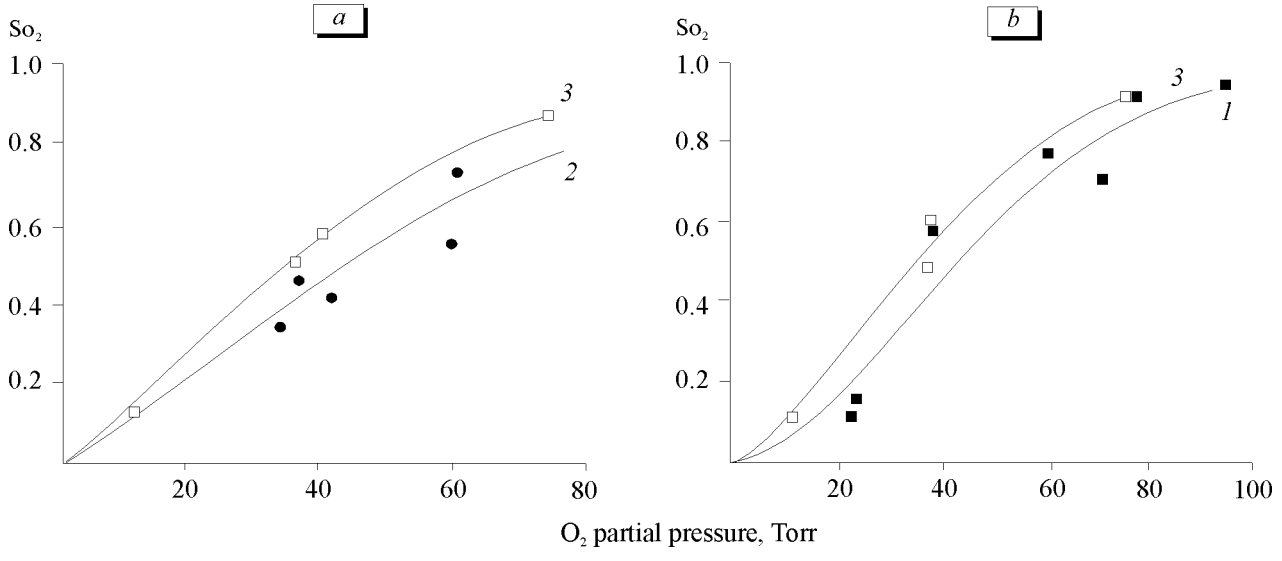

Fig. 3. Hemoglobin dissociation curves in individual blood samples. *1-3*: curves for dogs No. 1 (g=6.7 μ_B), 2 (g=5.4 μ_B), and 3 (g=5.1 μ_B), respectively.

Hem is known to form complexes with nitrogen bases with the formation of hemochromes [(Py)Hem(Py)], [(Im)Hem(Im)]. It also forms complexes with nitrogen bases and oxygen, such as [(O_2)Hem(Py)], [(O_2)Hem (Im)]. It is not surprising, therefore, that its *h*-form (Hem^h) can form the [(O_2)Hem^h(O_2)] complex, analogous to hemochromes, which can be called bis-(O_2)-hemochrome or bioxyhem. It is accepted that hemochrome is formed by simultaneous addition of two nitrogen bases [1]. It can be assumed, therefore, that the reaction of bioxyhem formation has the following appearance:

$$Hem^h+2O_2=[(O_2)Hem^h(O_2)]. \quad \textbf{(8)}$$

The concentration of bioxyhem in this reaction increases in proportion to the square of the dissolved oxygen concentration. This explains the presence of appreciable quantity of bioxyhemoglobin at Po_2>100 Torr and its degradation with O_2 release in the plasma at lower Po_2. Bioxyhem formation is accompanied by a decrease in pH [3] indicating that bioxyhem is less basic than Hem^h.

If the hypothesis on the magnetic field effect on hem-imidazole bonds is correct, the local action of the magnetic field of the approaching O_2 molecule (at a distance of 1 nm this field is 40 000 Gs) can initiate $r \leftrightarrow t$ transitions and cause the formation of *h*-form under natural conditions.

If this is the case, the magnetic field can be expected to affect the function of myoglobin and cytochromes.

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