

# Glycation of Leghemoglobin by Methylglyoxal in Comparison with Other Hemoglobins and the Influence on Peroxidase Activity

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**Abstract**—Nonenzymatic glycation is an irreversible posttranslational protein modification, which leads to a violation of physicochemical properties and functions. Glycation most often affects lysine and arginine residues. Since hemoglobins contain many lysine residues (average 9%), they are often targets for the glycating agents glyoxal and methylglyoxal (MG). A comparative study of the susceptibility for glycation of leghemoglobin (Lb) from bean nodules (*Vicia faba* L.), myoglobins (Mb) from sperm whale muscles and horse hearts, and hemoglobins (Hb) from bovine and human erythrocytes was carried out. The level of glycation was defined by the autofluorescence of protein-bound advanced glycation end products (AGEs). The glycation level of Lb was 2.5 times higher than that of sperm whale Mb and human Hb and five times higher than that of horse Mb and bovine Hb. The Lb glycation level depended on the presence of oxygen in the medium. Under microaerobic conditions, the amount of AGEs formed was three times lower than in an oxygen-containing environment, and the degradation of the heme group was also slower. Glycation also affected the peroxidase activity of hemoproteins. The initial rate of Lb peroxidase reaction was six times higher than that of myoglobins and 10–13 times higher than that of hemoglobins. Glycation decreased the rate of the Lb and hemoglobin peroxidase reaction, while for myoglobins it did not change or increased depending on the incubation time with MG.

**Keywords:** leghemoglobin, hemoglobin, myoglobin, methylglyoxal, non-enzymatic glycation, peroxidase activity

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## INTRODUCTION

Nonenzymatic glycation, which occurs through the Maillard reaction, is a widespread irreversible post-translational modification of proteins that leads to changes in their structure and properties. As a result, the functioning of proteins is disrupted; their solubility, stability and ability to undergo proteolytic degradation are reduced. Typically, nonenzymatic glycation occurs in the reaction of amino groups of proteins with active carbonyl compounds—reducing sugars and their metabolic products: glyoxal, methylglyoxal (MG), 3-deoxyglucosone, malondialdehyde, etc. [1]. The process begins with the formation of Schiff bases, followed by the Amadori rearrangement and the formation of stable protein adducts—advanced glycation end products (AGEs, Advanced Glycation End products). AGEs are a group of chemical compounds of various structures, including derivatives pyrrole, pyrazine, imidazole, and furan [2]. Most often found in proteins, modifications are carried out on lysine, arginine, and N-terminal amino acid residues [3, 4], and since hemoglobin proteins contain a large amount of lysine residues (on average, 9%), they are often a target for glycating agents: glyoxal and MG.

Many protein-bound AGEs exhibit high autofluorescence intensity when excited by far-UV light. Cross-linked AGEs formed with the participation of two lysine residues exhibit autofluorescence [5]. Deep UV-induced autofluorescence (deep-UV-induced autofluorescence, deep-UV-IAF) allows detection of a wide range of clinically significant AGEs, such as pentosidine ( $\lambda_{\text{isp}} \sim 380$  nm), argpyrimidine ( $\lambda_{\text{isp}} \sim 395$  nm), vesperlysin C ( $\lambda_{\text{isp}} \sim 405$  nm), vesperlysin A/B ( $\lambda_{\text{isp}} \sim 440$  nm), and arginine-derived AGEs ( $\lambda_{\text{isp}} \sim 525$  nm) [6]. The accumulation of AGEs is an indicator of many diseases [7].

The most active glycating agent is  $\alpha$ -ketoaldehyde methylglyoxal (MG) [8]. There are many pathways for the formation of this dicarbonyl compound, but it is most commonly formed as a by-product of glucose metabolism in the glycolytic pathway [9]. The formation of MG in prokaryotic and eukaryotic cells is described in reviews [10, 11]. The situation when dicarbonyl compounds accumulate as a result of their increased production and/or decreased detoxification is called dicarbonyl stress [1, 12]. This condition accompanies metabolic and age-related diseases, in

which oxidative and inflammatory reactions are enhanced [7].

Dicarbonyls are involved in the development and exacerbation of diabetes, atherosclerosis, obesity, cardiovascular diseases, renal failure, neurological disorders, and cancer [4, 12, 13]. It is believed that MG is responsible for the dysfunction of micro- and macrovessels in diabetes mellitus [8, 12]. The use of MG as a new biomarker for the diagnosis of diabetes mellitus is discussed [8], but hydroimidazolone AGEs derived from arginine, which accumulate in aging and dysfunctional tissues, have been proposed to be considered as markers of the progression of various diseases [1, 4].

Hemoglobin proteins have a high tendency to glycation, since they contain accessible lysine and arginine residues. Due to their long lifespan, red blood cell hemoglobins are accumulators of AGE. Note that hemoglobin proteins form a superfamily. Characteristic representatives of this superfamily are hemoglobin (Hb), myoglobin (Mb), and leghemoglobin (Lb). Leghemoglobin is a plant hemoglobin synthesized in the nitrogen-fixing nodules of leguminous plants, where it functions as an oxygen buffer. In structure and properties, Lb is similar to myoglobin. Unlike Mb and Hb, glycation of Lb has been virtually unstudied. The objective was to study the glycation of Lb by methylglyoxal in comparison with hemoglobins and myoglobins. Since hemoglobin proteins exhibit peroxidase activity (often called pseudoperoxidase to distinguish it from true peroxidase activity), the effect of glycation on their ability to reduce hydrogen peroxide has also been studied.

## MATERIALS AND METHODS

The following reagents were used in this work: hemoglobins from bovine and human erythrocytes, myoglobins from sperm whale skeletal muscles and horse hearts, methylglyoxal, hydroperoxide *tert*-butyl (*t*-BOOH), *o*-dianisidine dihydrochloride, pyridine, sodium ascorbate, sodium dithionite, methylglyoxal, polyacrylamide (PAAG), sodium dodecyl sulfate (SDS), Coomassie Brilliant Blue R-250 (Sigma-Aldrich, United States), phenylmethylsulfonyl fluoride (PMSF) (AppliChem, Germany), Tris (ICN, United States), and Toyopearl HW-50 Fine, Toyopearl 650 M DEAE (Toyo Soda, Japan). The remaining reagents are from Reakhim (Russia), chemically pure or especially pure grade.

**Isolation and purification of leghemoglobin from bean nodules.** Lb was isolated from root nodules of beans (*Vicia faba* L.) of the varieties Russian Black (sometimes the name *Faba bona* Medik is used) according to the method in [14]. Plants were grown in field conditions in Moscow region (Russia), the nodules were collected at the budding stage or the beginning of flowering, separated from the plants, and filled

with cold 100 mM K-phosphate buffer (pH 7.2), containing 200 mM sodium ascorbate, 1% high molecular weight polyvinylpyrrolidone, 50  $\mu$ M PMSF, and 1 mM  $\text{MgCl}_2$ . The buffer to nodule ratio was 3 : 1 by weight. The nodules were thoroughly ground in an agate mortar, after which the homogenate filtered through two layers of nylon cloth. The filtrate was centrifuged with cooling for 5 min at 1000 *g* to remove contaminants and large particles of plant tissue from the extract. The Lb-containing fraction was obtained by salting out the proteins of the supernatant with  $(\text{NH}_4)_2\text{SO}_4$  in the saturation range of 40–80%. The pellet containing Lb was dissolved in a minimal amount of 20 mM K-phosphate buffer (pH 7.2) and centrifuged at 23000 *g* for 20 min, and the supernatant was then applied to the column (2  $\times$  43 cm) with the Toyopearl HW-50 Fine equilibrated with 25 mM Tris-HCl (pH 7.5) with 200 mM NaCl. To suppress protease activity, PMSF was added to the buffer to a final concentration of 10  $\mu$ M.

Subsequent purification of Lb was carried out on an anion exchanger Toyopearl 650M DEAE on a 2  $\times$  4 cm column, equilibrated with 25 mM Tris-HCl buffer (pH 7.5). Lb fractionation was performed using a linear gradient with NaCl from 0 to 0.5 M. The elution rate was 2.13 mL/min. The Lb fraction exited the column at a NaCl concentration of 0.2–0.4 M. The eluate collected from the ion exchange column was oxidized with potassium ferricyanide and dialyzed against 25 mM K-phosphate buffer, pH 7.2. The protein was concentrated on a Millipore membrane (Merck, Germany) in an ultramembrane concentration cell under an argon flow. All procedures were carried out at a temperature of 4°C. The concentration of Lb was determined using the millimolar extinction coefficient  $\epsilon_{525} = 9.52 \text{ mM}^{-1} \text{ cm}^{-1}$ , since the wavelength of 525 nm in the spectra of metLb and oxyLb corresponds to the isosbestic point.

**Obtaining oxygenated hemoglobin proteins.** To obtain oxygenated hemoglobin proteins, crystalline sodium dithionite, which reduces hemoproteins, was added to a 0.6 mM protein solution. The protein solutions were then dialyzed for 15 h at 4°C against 50 mM K-phosphate buffer (pH 7.2). The oxygenated form was recorded by characteristic maxima in the optical absorption spectrum.

**Nonenzymatic glycation of hemoglobin proteins.** MG was used to carry out the non-enzymatic glycation reaction. The reaction mixture contained 0.06 mM oxygenated hemoproteins in 50 mM K-phosphate buffer, pH 7.4, 5 mM MG (protein : MG = 1 : 80 mM). Incubation was carried out at 37°C for 96 h.

In the case when the Lb reaction mixture contained 0.24 mM oxyLb in 25 mM K-phosphate buffer, pH 7.4, 12 mM MG (Lb : MG = 1 : 50). Incubation was carried out at 37°C for 120 h. When studying the influence of oxygen to create microaerobic conditions,

flasks with Lb solution were purged with special-purity grade gaseous nitrogen (oxygen volume fraction no more than 0.0005%) for 15 min.

Advanced glycation end products were measured by autofluorescence at an excitation wavelength of 320 nm and an emission wavelength of 450 nm. The measurements were carried out using a spectrophotometer. RF-5302 PC (Shimadzu, Japan) in a 0.5 mL microcuvette at a medium scanning speed, high sensitivity (according to the device marking), excitation light slit width of 5 nm, and emitted light of 10 nm. Before measurement, the protein solution was diluted ten times.

**SDS-electrophoresis in PAGE.** Electrophoresis was performed in 12% PAAG blocks of the size  $15 \times 15 \times 2$  mm using the Laemmli method. Samples of Lb, prepared as described above, were predialyzed in K-phosphate buffer to separate low molecular weight substances, then the sample buffer was added in a ratio of 1 : 1, heated for 5 min at 95°C, and applied to the gel. The sample buffer was prepared based on 0.1 M Tris-HCl buffer (pH 6.8) with 4% SDS, 0.2% bromophenol blue, and 20% glycerol. 20  $\mu$ L of protein solution was applied to the gel. 0.2 M Tris-glycine buffer (pH 8.3) containing 0.1% SDS was used as the electrode buffer. After completion of the protein separation process, the gel was fixed and stained with Coomassie Brilliant Blue R-250 solution. Electrophoresis was carried out at 4°C and  $I = 50$  mA,  $U = 150$  V. The Elf-4 power supply (Helikon, Russia) was used.

**Determination of peroxidase activity.** The peroxidase activity of proteins was determined spectrophotometrically by measuring hydroperoxide-dependent oxidation of *O*-dianisidine according to the method in [15]. The composition of the reaction mixture was 80 mM K-phosphate buffer (pH 7.2), 1 mM *O*-dianisidine, and 3  $\mu$ M hemoprotein. The reaction was initiated by the addition of *t*-BOOH to a final concentration of 4.7 mM. Formation of the oxidation product of *O*-dianisidine-bisazobiphenyl was recorded spectrophotometrically at 450 nm at 25°C for 10 min with an interval of 10 s. An extinction coefficient of  $30 \text{ mM}^{-1} \text{ cm}^{-1}$  was used to calculate the concentration of the product. Peroxidase activity was expressed as  $\mu$ moles of bisazobiphenyl formed per minute.

**Spectrophotometric measurements.** The optical absorption spectra were recorded in the range from 220 to 700 nm on a UV-VIS spectrophotometer Cary 300 (VarianBio, United States) at room temperature in a cuvette ( $V = 0.5$  mL) with an optical path length of 1 cm at a scanning speed of 600 nm/min.

All experiments were performed at least in triplicate. Data are presented as mean values  $\pm$  standard deviation. Statistical processing of data was carried out based on 3–5 analytical replicates in the Microsoft Excel mode.

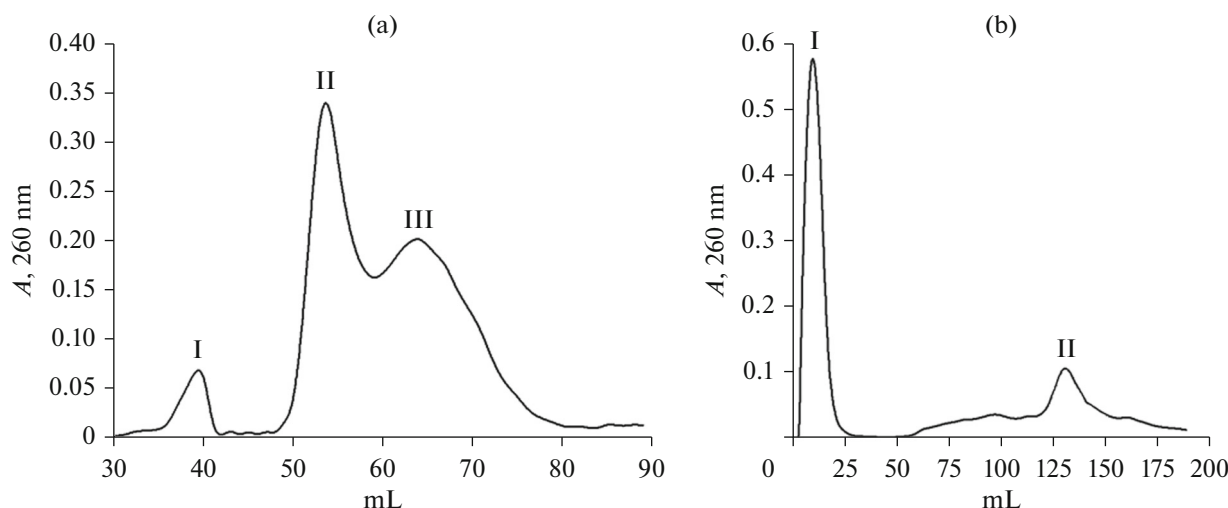
## RESULTS AND DISCUSSION

**Isolation of leghemoglobin from bean nodules.** The present study aimed to compare the susceptibility to glycation Lb with other hemoglobin proteins (myoglobins and hemoglobins). For this purpose, a purified preparation of Lb from bean nodules was needed. Lb was isolated using standard methods [14], which included purification by gel filtration on Toyopearl HW-50 Fine and ion exchange chromatography on Toyopearl 650 M DEAE. These media gave the best results in Lb purification. The protein elution profile on the Toyopearl HW-50 Fine column had three peaks (Fig. 1a), with Lb emerging as the second. In ion exchange chromatography, Lb emerged as the first peak (Fig. 1b).

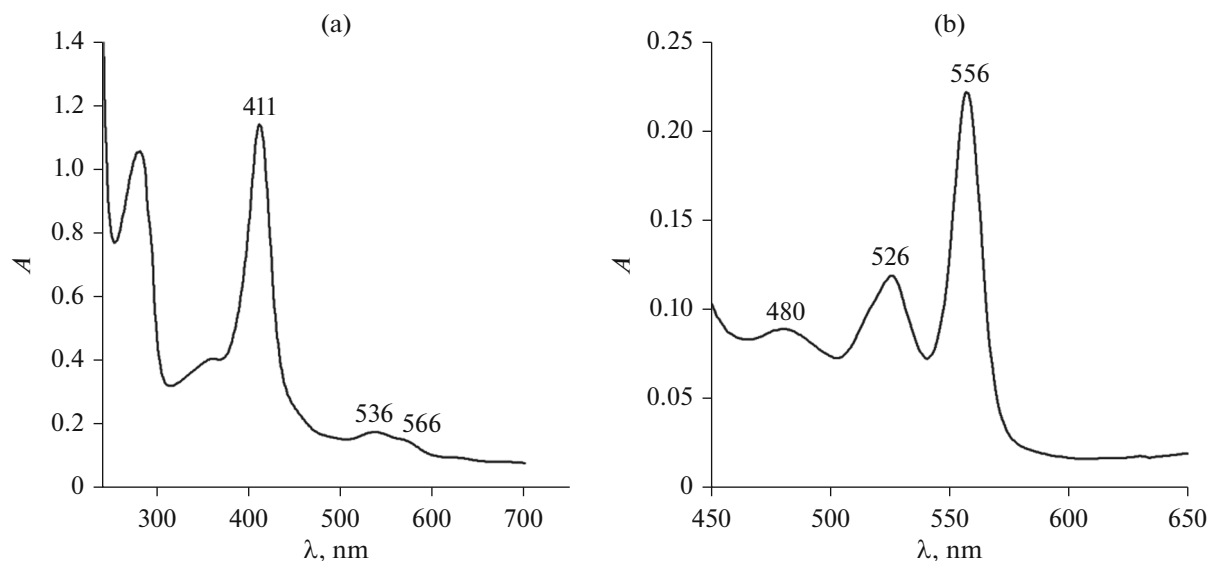
The Lb obtained in this way was characterized by UV-visible optical absorption spectra and spectra of the pyridine–hemochrome complex. The character of the spectra and absorption maxima (411, 536, and 566 nm) corresponded to the oxygenated form of Lb (Fig. 2a). The spectrum of the reduced Lb complex with pyridine in an alkaline solution also had absorption maxima (480, 526, and 556 nm) characteristic of the native unmodified protein (Fig. 2b).

**Modification of leghemoglobin by methylglyoxal: Influence of oxygen conditions.** Unlike hemoglobins and myoglobins, nonenzymatic glycation of Lb has previously been virtually unstudied. A fraction of glycated Lb was previously isolated from cells of *E. coli*, in which it was expressed [16]. In the present work, nonenzymatic glycation of Lb with methylglyoxal was carried out in experiments in vitro. The reaction proceeded under two variants of oxygen conditions for 120 h (five days). Figure 3 shows the results of SDS-electrophoresis in 12% PAGE of leghemoglobin collected at different times of incubation with MG. With an increase in the degree of glycation of Lb, there was a decrease in the proportion of dimeric and tetrameric forms of the protein with a simultaneous increase in the number of high-molecular oligomeric forms. A similar picture was observed in experiments with sperm whale Mb.

In this case, protein aggregation occurred with the release and/or destruction of the heme group, which is also evidenced by the optical absorption spectra (Fig. 4). During incubation with MG, absorption in the Soret band region (418 nm) decreased and absorption at 280 nm increased. Moreover, the decrease in absorption in the Soret band was more pronounced in an oxygen-containing environment, which indicates that, under these conditions, active forms of oxygen are formed that destroy the heme group of the protein. The formation of the superoxide anion radical has previously been reported in the glycation reaction of amino acids and proteins [17, 18]. In contrast, UV absorption was higher in the microaerobic environment. The changes were especially noticeable after 120 h of incubation. Measurement of protein autoflu-



**Fig. 1.** Elution profile of bean root nodule cytosol proteins on a Toyopearl HW-50F column: I, proteins with MM > 110 kDa, II, proteins with MM 90–60 kDa; III, proteins with MM 60–15 kDa (a); elution profile of fraction II proteins on the column Toyopearl 650 M DEAE using the NaCl concentration gradient 0–0.5 M (b).

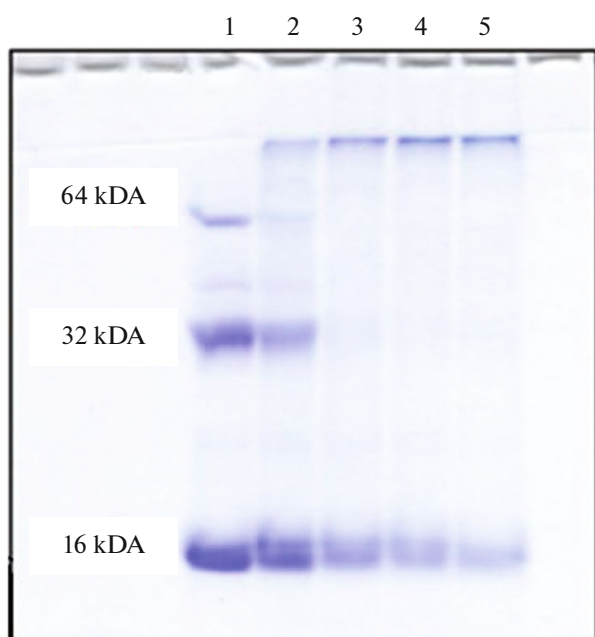


**Fig. 2.** (a) Optical absorption spectra of Lb isolated from bean nodules in K-phosphate buffer, pH 7.2; (b) spectra of reduced Lb in an alkaline pyridine solution.

orescence also showed that protein-bound AGEs were formed in the presence of oxygen (Fig. 5). The results obtained for Lb are also characteristic of other similar proteins, since they reflect the features of the nonenzymatic glycation reaction. This reaction is highly dependent on the conditions of the reaction: the presence of metals, temperature, pH, and oxygen conditions [7]. Since glycation processes are coupled with oxidation processes, the term “glycooxidation” is often used to describe this post-translational modification.

**Peroxidase activity of hemoglobin proteins modified with methylglyoxal.** It is known that nonenzymatic gly-

cation leads to changes in the enzymatic activities of hemoglobin proteins [19–22]. We studied the effect of glycation by methylglyoxal on the peroxidase activity of Lb from bean nodules, Mb from sperm whale and horse muscles, and Hb from human and bovine erythrocytes. Hemoproteins were incubated with MG in an oxygen-containing medium for 96 h (four days) at 37°C. The degree of modification was defined by the intensity of autofluorescence of the AGEs formed on the protein (Fig. 6). Under the same conditions, Lb was glycated to the greatest extent.



**Fig. 3.** Electrophoresis of Lb 12% SDS-PAAG after incubation with methylglycol in the ratio oxyLb : MG = 1 : 80 mM for (1) 0, (2) 6, (3) 24, (4) 48, and (5) 72 h.

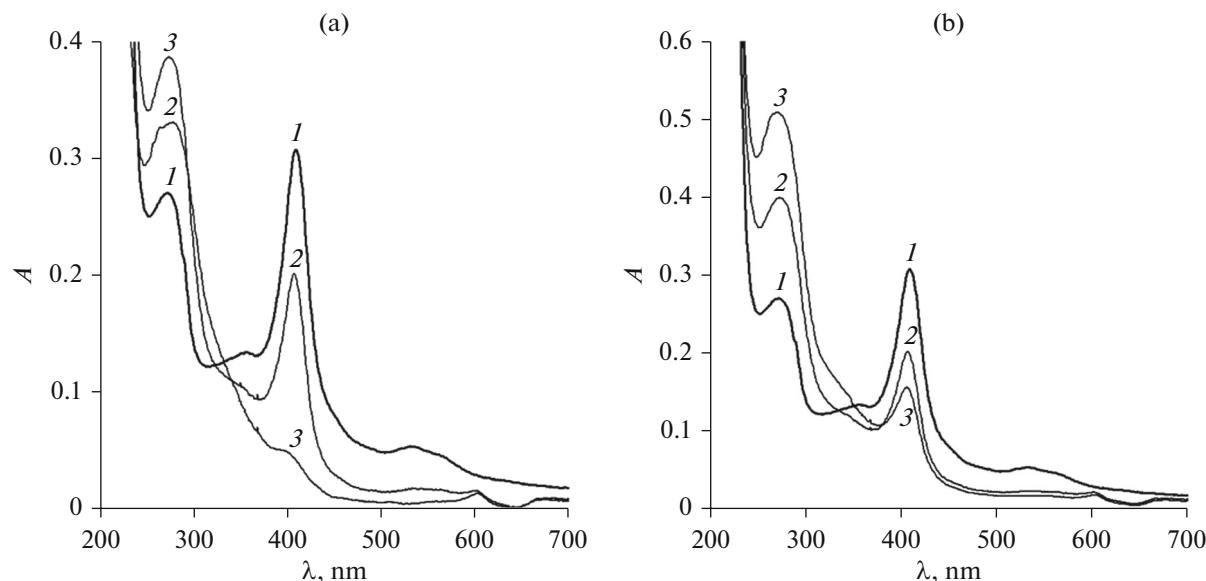
During the incubation of proteins with MG, samples were taken every 24 h to define the peroxidase activity, which was measured in the presence of t-BOOH and *O*-dianisidine. Figure 7 shows the change in peroxidase activity of the hemoproteins studied. Glycation of Lb and hemoglobins resulted in a decrease in the initial rate of the peroxidase reaction.

By 96 h of incubation, the activity of glyated Lb (GLb) was approximately two times lower than in the unmodified protein. Similar changes were observed for human GHb. In the bull GHb, activity was 35% lower. The lowest activity of GLb was noted at 96 h of incubation, for human GHb at 72 h, for bovine GHb at 48 h. In this case, the peroxidase activity of Mb either did not change or increased slightly.

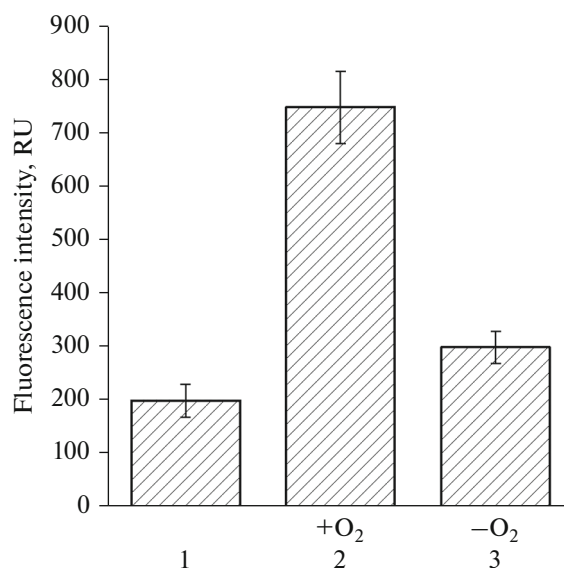
**Discussion of the results.** The first protein for which nonenzymatic glycation was demonstrated was hemoglobin. Hb is a long-lived protein; its lifetime in the erythrocyte is  $120 \pm 20$  days. This is enough for glucose to react with the amino group of the N-terminal valine residue  $\beta$ -subunits and form a stable complex (HbA1c). Since the HbA1c level reflects the blood glucose level over a period of 4–6 weeks, it is used as a molecular marker in the diagnosis of diabetes mellitus [23].

In addition to sugars,  $\alpha,\beta$ -dicarbonyl compounds can enter into the nonenzymatic glycation reaction—glyoxal and methylglyoxal, the glycation activity of which exceeds by 20000 times the activity of glucose [9]. MG deserves special attention, because it reacts with amino and imidazole groups of amino acid residues of proteins, forming covalently linked AGEs: carboxyethyllysine, carboxymethyllysine, imidazolones, argpyrimidines, and cross-linked dimers of lysine with methylglyoxal (MOLD—methylglyoxal-derived lysine dimers) [9, 24]. Such modifications are irreversible and lead to changes in the structure, conformation, and charge, and, as a consequence, to dysfunction of the protein [25].

Glycation is the most well studied in vitro by methylglyoxal of the human erythrocyte Hb (HbA0). In works [3, 26, 27] the influence of MG on its structure



**Fig. 4.** Optical absorption spectra of Lb, treated with methylglyoxal (a) in an oxygen-containing environment and (b) under microaerobic conditions, during (1) 48, (2) 72, and (3) 120 h of reaction.

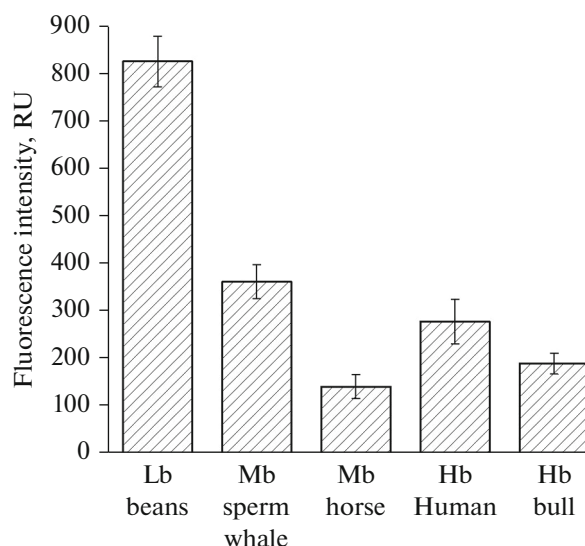


**Fig. 5.** Fluorescence intensity of AGEs associated with Lb after incubation with MG for 120 h: initial protein solution (1) without MG, (2) with MG in the presence of oxygen + O<sub>2</sub>, and (3) with MG in an oxygen-free environment – O<sub>2</sub>.

and function was characterized. Compared to native Hb, glycated (GHbA0) had lower absorbance at 280 nm, reduced tryptophan fluorescence, increased  $\alpha$ -helix content, a less hydrophobic surface, and a stable quaternary structure. Release of iron from heme by H<sub>2</sub>O<sub>2</sub> and subsequent oxidation at GHbA0 proceeded less intensively. GHbA0 was also thermally more stable. The modification sites and the nature of the formed AGEs were identified.

The peroxidase activity of glycated GHbA0 was lower than that of HbA0. In works [3, 26] it was shown that, when human Hb is modified with methylglyoxal, mainly hydroimidazolone derivatives of arginine residues are formed. The degree of modification of these residues depended on their accessibility to the solvent. According to the data of work [28], in Hb treated with MG, the lysine and arginine residues were modified. Some of these modifications have been identified in Hb isolated from human blood [28]. In patients with poorly controlled type-2 diabetes mellitus, the levels of carboxymethylated lysine and arginine derivatives with hydroimidazole were significantly higher than in healthy individuals. Thus, certain Hb modifications may be markers for assessing protein damage by methylglyoxal in the body [26]. Lys-66 $\beta$ , located in the heme pocket, is very easily glycated. Since this lysine is involved in oxygen binding, its modification may be the cause of a decrease in the oxygen-binding properties of Hb [29] and a change in its inherent enzymatic activities.

The influence of MG on changes in the structural and physicochemical properties of myoglobins was also studied [30–33]. A study of Mb glycated with glu-

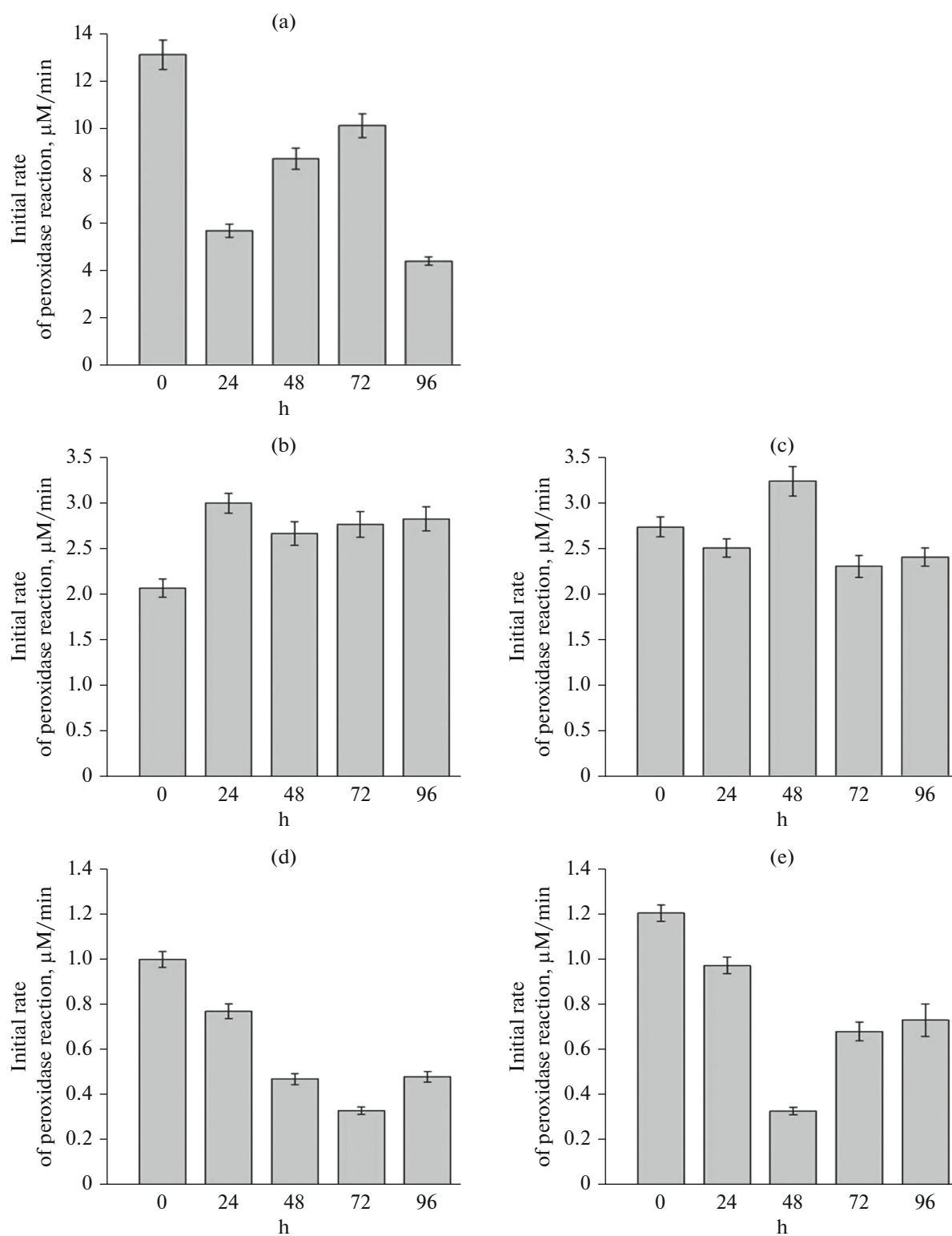


**Fig. 6.** Fluorescence intensity of AGEs bound to different proteins after incubation with MG for 96 h.

cose, D-ribose, 3-deoxyglucosone, and MG showed that AGEs, as in the case of Hb, are most often formed in lysine and arginine residues. In the reaction of Mb with MG, about half (56%) of all lysine residues undergo modification [34]. Glycation of lysine residues results in the formation of bis(lysyl)imidazolium cross-links. Long-term incubation of Mb with MG causes heme release, change in tryptophan fluorescence, reduction of  $\alpha$ -helix, and increase in the  $\beta$ -sheet content [31, 33, 34]. These changes cause formation of amyloid-like aggregates of Mb.

For equine heart Mb, it was also shown that the main glycation sites are lysine and arginine residues, and that with an increase in incubation time to 14 days, additional AGEs are formed. Mass spectrometric studies revealed modifications at two specific lysine residues: Lys-87 and Lys-133 [32]. Erythrocyte Hb, myoglobin, and Lb are “lysine-rich proteins” in which the number of lysine residues ranges from 7 to 12 per protein subunit or molecule. With increasing incubation time with MG, there was a gradual decrease in the proportion of  $\alpha$ -helices [32]. In addition, MG modification affected the tertiary structure and hydrophobicity of the protein. It has been shown that GMb had higher electrophoretic mobility in PAGE and increased optical absorption in the 280 nm region [30].

Lysine residues are positively charged basic amino acids and are often located on the surface of proteins. Due to their high pKa values, they create stable electrostatic and ionic interactions [35], which ensures the stability and solubility of the protein molecule [35, 36]. For example, Mb of diving mammals has a large positive charge, which causes electrostatic repulsion of protein molecules and makes the protein soluble even



**Fig. 7.** Peroxidase activity of methylglyoxal-modified hemoglobin proteins: (a) Lb from bean nodules, (b) Mb from sperm whale muscles, (c) Mb from horse heart, (d) Hb from human erythrocytes, and (e) Hb from bovine erythrocytes.

at very high concentrations in muscle cells [37]. The function of lysine in Lb has not been discussed in the literature. By analogy with Mb, it can be assumed that lysine residues in Lb increase its solubility, the concentration of which in the cytosol of plant cells is quite high (0.1–0.5 mM) [38].

It is assumed that glycation of lysine residues in Lb can occur both in the natural environment (in a plant cell) and in an expression system (bacteria, yeast) [18]. Glycation may be the cause of decreased Lb solubility and the formation of covalently cross-linked aggregates, which leads to a decrease in the efficiency of nitrogen fixation in nodules and the formation of inclusion bodies during expression of recombinant Lb. It is known that some Lysine residues in proteins can function as glycation hotspots, through which protein function and lifespan are regulated [39]. The availability of such hot spots were shown in the plant and rhizobial proteomes of bean root nodules (*Phaseolus vulgaris* L.) [40].

The most well-known function of hemoglobin proteins is the storage and transport of O<sub>2</sub>, but they can also exhibit a number of enzymatic activities [41], including esterase [42] and peroxidase [14, 43]. It was the peroxidase activity that was first described for these proteins [44], and in [45] the peroxidase (pseudo-peroxidase, as the authors of the article called it) activity of soybean Lb was described in comparison with sperm whale Mb. The maximum velocity of peroxidase reaction with guaiacol for Lb was seven times greater than for Mb. In the present work, the peroxidase reaction rate of Lb was approximately six times higher than that of myoglobins and 10–13 times higher than that of hemoglobins. This is due to a number of structural features of Lb. These include the hydrophobic environment of the heme, the mobility of the imidazole ring of the distal histidine, the lability of the porphyrin, and the presence of arginine and asparagine residues that bind and orient the substrates.

Glycation in vitro leads to an increase in the esterase activity of Hb and Mb due to an increase in the affinity of the protein for the substrate and the reaction rate [21]. Greater esterase activity has also been shown for HbA1c compared to HbA0 [21]. In contrast, glycation reduced the peroxidase activity of HbA0 [19, 22]. Moreover, glycated horse heart Mb exhibited greater peroxidase activity than native, which correlated with the formation of ferrylmyoglobin in the reaction with H<sub>2</sub>O<sub>2</sub> [20].

Glycation can lead to the accumulation of misfolded proteins that aggregate to form amyloid-like structures that are deposited in the intercellular space and inside the cell in various diseases [46]. Erythrocyte Hb, as a long-lived protein, is particularly prone to such aggregation. Modification with glyoxal, transforming  $\alpha$ -helices into  $\beta$ -sheets, enhances the amyloidogenic potential of Hb [47–49]. Because glycation results in the release of heme and iron from proteins,

glycated Hb and Mb pose a serious threat when they enter the bloodstream [20, 48, 50]. Such iron is involved in the production of free radicals in the Fenton reaction, which increases oxidative stress, especially in diabetics [20, 48]. Glycation accelerates the autooxidation of oxyMb, resulting in the formation of a superoxide anion radical [20]. Its formation can be catalyzed by cation-radicals of Schiff bases localized on glycated proteins, which act as electron donors [17, 18].

Studying the glycation process will help to understand better the molecular mechanisms of obesity, diabetes, cardiovascular and neurological diseases, renal failure, aging, and cancer, in which the proportion of glycated proteins increases. Identifying the features of this process is also important for biotechnology. In particular, the results obtained may be useful for developing an effective technology for producing stable and soluble recombinant hemoglobins, including Lb, for example, in the production of so-called “plant meat.” Foreign proteins can undergo post-translational modifications, among which nonenzymatic glycation occupies a prominent place [16]. This affects both the properties of proteins and the state of the expression system. It has been shown that the expression of the Lb gene in cells *E. coli* affects the state of the bacterial cell under oxidative and nitrosative stress [51], and since recombinant Lb can be glycated [16], studying this process will help to increase the stability of the expression system.

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## ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This work does not contain any studies involving human and animal subjects.

## CONFLICT OF INTEREST

The authors of this work declare that they have no conflicts of interest.

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