The results of this investigation, showing the stimulating effect of protamine in the early stages of HChE and its regulatory action on blood serum lipoproteins during long-term HChE, suggest that protamine and alkaline proteins similar to it in structure possess antiatherogenic properties.

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MECHANISM OF HYDROGEN PEROXIDE INDUCED OXIDATION OF OXYHEMOGLOBIN

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UDC 536.6:[577.158.7+546.215]

KEY WORDS: hemoglobin; hydrogen peroxide; oxidation.

Autooxidation of oxyhemoglobin (HbO_2) into methemoglobin (MtHb) is accompanied by the formation of superoxide anion-radicals [3, 11], from which H_2O_2 is formed by spontaneous dismutation, and also under the influence of superoxide dismutase (SOP) [9]. Both O_2 and H_2O_2 can induce oxidation of hemoglobin, but there is reason to suppose that H_2O_2 has the most powerful destructive action on hemoglobin [6, 11].

Incidentally, in the literature on the study of oxidative destruction of hemoglobin through the action of H_2O_2, most attention has been paid to direct interaction of the active center of hemoglobin with H_2O_2 [6, 7]. Yet hydrogen peroxide can oxidize the amino groups of proteins, including SH-groups [14], and this can cause structural damage to the protein

N. I. Pirogov Second Moscow Medical Institute. Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 112, No. 7, pp. 46-49, July, 1991. Original article submitted July 24, 1990.

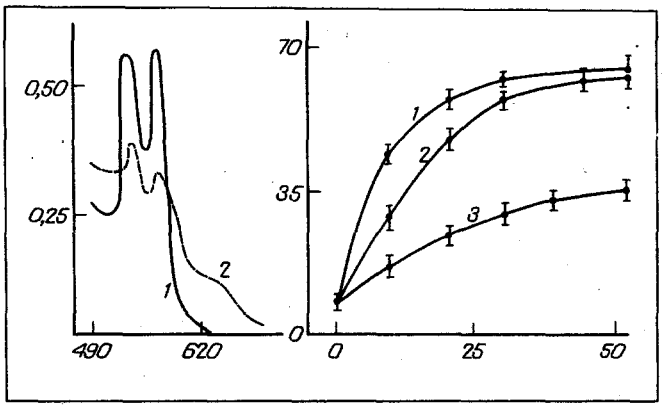

Fig. 1 Fig. 2

Fig. 1. Absorption spectra of rat HbO_2 before and after addition of H_2O_2. To reaction medium containing $1.2 \cdot 10^{-5}$ M HbO_2 was added H_2O_2 in a concentration of $2.8 \cdot 10^{-7}$ M; to a cuvette containing 9.8 ml of HbO_2 solution was added 0.2 ml of $1.4 \cdot 10^{-5}$ M H_2O_2. 1) Before addition of H_2O_2, 2) 50 min after addition of H_2O_2. Samples not containing H_2O_2 and also samples containing 10^{-8} M catalase, to which H_2O_2 was added in a concentration of $2.8 \cdot 10^{-7}$ M, were used as the control. Their spectra remained unchanged for 50 min. Abscissa, wavelength (in nm); ordinate, optical density.

Fig 2. Kinetic curves of oxidation of rat HbO_2 by different initial concentrations of H_2O_2. Initial concentrations of H_2O_2: 1) $7 \cdot 10^{-7}$ M; 2) $3.5 \cdot 10^{-7}$ M; 3) $1.4 \cdot 10^{-7}$ M Initial HbO_2 concentration $1.2 \cdot 10^{-5}$ M. Here and in Fig. 3: ordinate, MtHb concentration (in %); abscissa, time (in min).

molecule. We know that damage to certain amino groups of globin leads to stimulation of autooxidation – spontaneous oxidation into MtHb following interaction with O_2 [4, 11], and as a result of this, as was pointed out above, O_2 and H_2O_2 are reformed. Thus in the absence of enzymes decomposing hydrogen peroxide, it may be formed constantly in a system containing oxidized HbO_2, thereby influencing the oxidation process itself.

The aim of this investigation was to study oxidation of HbO_2 under the influence of low concentrations of H_2O_2 (10^{-7} M), almost two orders of magnitude lower than the concentration of HbO_2. Under those conditions, H_2O_2 initiates autooxidation of hemoglobin.

EXPERIMENTAL METHOD

The following reagents were used: NaCl and KH_2PO_4 from "Reakhim" (chemically pure), NaN_3 and L-histidine from "Reanal," Hungary; *o*-phenanthroline from "Chemapol," Czechoslovakia; catalase and α-tocopherol from "Serva," West Germany. The mother solution of H_2O_2, in a concentration of 14.7 M (from "Reakhim," chemically pure) was diluted to the required concentrations with 20 mM KH_2PO_4 solution, pH 7.4. The concentration of the mother solution of H_2O_2 was determined by titration with potassium permanganate [2]. The 10^{-3} M mother solution of α-tocopherol was prepared on double-distilled ethanol. Solutions of all the other reagents were made up in 20 mM KH_2PO_4, pH 7.4.

To prepare hemoglobin solution free from other metal-containing proteins, erythrocytes of noninbred rats obtained by the method in [10] were hemolyzed osmotically by incubation for 20 min with 5 mM KH_2PO_4, pH 7.4, in the ratio of 1:19 by volume. The hemolysate was subjected to successive gel-filtrations on Sephadexes G-150 and G-25 ("Pharmacia," Sweden) to purify it from catalase and SOD respectively, and also from low-molecular-weight compounds. The absence of catalase as an impurity in the hemoglobin fraction was verified colorimetrically [1]. To eliminate any possible effect of contamination by bivalent cations, the eluting solution for gel-filtration contained $5 \cdot 10^{-4}$ M *o*-phenanthroline. It was present in the same concentration in the reaction medium in all experiments.

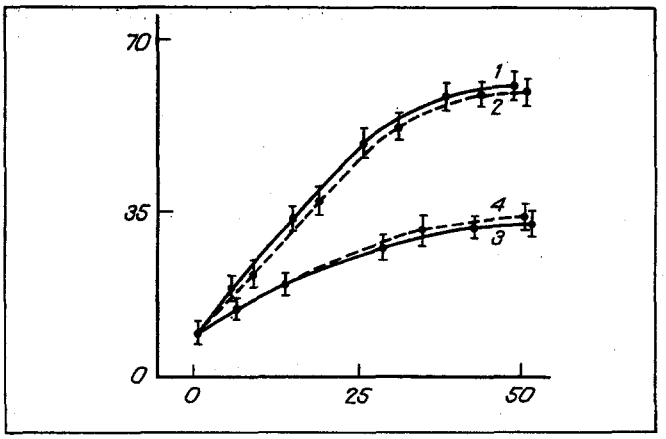

Fig. 3. Oxidation of rat HbO_2 by H_2O_2, using different concentrations of reagents but in the same relative proportions: 1) $1.2 \cdot 10^{-5}$ M HbO_2 and $2.8 \cdot 10^{-7}$ M H_2O_2; 2) $2.4 \cdot 10^{-5}$ M HbO_2 and $5.6 \cdot 10^{-7}$ M H_2O_2; 3) $1.2 \cdot 10^{-5}$ M HbO_2 and $1.4 \cdot 10^{-7}$ M H_2O_2; 4) $2.4 \cdot 10^{-5}$ M HbO_2 and $2.8 \cdot 10^{-7}$ M H_2O_2.

The spectrophotometric measurements were made on an SF-14 differential spectrophotometer The continuous spectrum was recorded from 490 nm to 750 nm in the course of 84 sec. The measurements were made in a cylindrical cuvette with vertical passage of the beam of light by means of an Ulbricht's sphere, reducing scattering of light. The length of the optical path was 3 cm.

The hemoglobin concentration in moles of heme-groups was determined assuming the coefficient of absorption at 540 nm to be $1.5 \cdot 10^4$ $M^{-1} \cdot cm^{-1}$ [4]. The MtHb concentration was determined from the ratio between the maxima of absorbance of light, by the method of Salvati and co-workers [13].

Deoxygenation of hemoglobin was carried out by creating a vacuum with a pressure of 10^{-1} atm. The apparatus consisted of a vacuum cuvette, connected to a vacuum pump, to which a regulating valve was connected parallel with the cuvette, to discharge the vacuum. The hemoglobin solution was introduced into a cuvette which had a separate isolated compartment containing H_2O_2. When 30 sec had elapsed after application of the vacuum the cuvette was upturned and vigorously shaken. In this way the H_2O_2 solution was mixed with the hemoglobin solution.

EXPERIMENTAL RESULTS

The spectrum of the HbO_2 solution before and after incubation with H_2O_2 is shown in Fig. 1. During incubation a change took place in the spectrum, corresponding to MtHb accumulation [8]. The MtHb, moreover, accumulated in amounts many times greater than the quantity of H_2O_2 added initially (Fig. 2). This was true for hemoglobin both in erythrocytes and in their hemolysates, provided that catalase was inhibited beforehand with 15 mM NaN_3.

It is an interesting fact that the initial velocity of oxidation of hemoglobln within the range of concentrations of reagents tested was directly proportional to the initial H_2O_2 concentration provided that the initial HbO_2 concentration was constant. In the case of a simultaneous increase or decrease in the concentrations of both reagents the rate of increase of the methemoglobin concentration remained virtually unchanged (Fig. 3).

The presence of catalase prevents MtHb accumulation. If catalase was present in the reaction medium before addition of H_2O_2, the process of hemoglobin oxidation was completely absent. Addition of catalase a short time after addition of H_2O_2 also arrested the further oxidation of hemoglobin (Table 1). It will be noted that the quantity of hemoglobin oxidized by the time of addition of catalase was many times higher than the quantity of H_2O_2 added initially.

Another interesting fact is that well-known antioxidants such as α-tocopherol in a concentration of 10^{-5} M and L-histidine in a concentration of $3 \cdot 10^{-3}$ had no action on HbO_2 oxidation in the presence of H_2O_2, whereas these antioxidants gave a small (about 17%) but significant protective effect on HbO_2 oxidation induced by 0.65% glutaraldehyde at pH 5.9.

TABLE 1. Inhibition by Catalase of H_2O_2-Induced Oxidation of HbO_2

Experimental conditions	Time after addition of H_2O_2, min		
	0	concentration of MtHb, %	60
Control	7,5±0,20	30,0±2,00	62,0±2,20
14 min after addition of H_2O_2	7,5±0,25	28,0±2,10	28,0±1,40
Before addition of H_2O_2	7,5±0,20	7,5±0,25	7,5±0,20

Legend. To 12 μM HbO_2 were added 0.28 μM H_2O_2 and 10^{-8} M catalase. Here and in Tables 2 and 3 mean values from four experiments ± the error of the mean are shown.

TABLE 2. Oxidation of Rat Hemoglobin by H_2O_2 in the Presence and Absence of Oxygen

Serial No.	Exposure of H_2O_2 to vacuum		Concentration of MtHb, %	
			before H_2O_2 exposure and its addition	after 7 min incubation with H_2O_2
1	+	—	7,0±0,4	7,7±0,8
2	—	—	7,0±0,4	7,0±0,4
3	+	+	7,1±0,5	7,2±0,4
4	—	+	7,0±0,5	64,0±2,1

Legend. 10 ml of 12 μM rat hemoglobin was introduced into a vacuum cuvette. Under a vacuum, 0.6 ml of $1.4 \cdot 10^{-5}$ M H_2O_2 was added to the hemoglobin solution. Initial H_2O_2 concentration in reaction medium 0.84 μM. 7 min after addition of H_2O_2, air was allowed into the cuvette. 7 sec after opening of the inlet valve, 0.1 ml of 10^{-6} M catalase was added to the reaction mixture to prevent further oxidation of hemoglobin, and the samples were subjected to photometry. Samples 2 and 4, not exposed to a vacuum also were incubated in the vacuum cuvette.

To determine the role of oxygen in the reaction of HbO_2 with H_2O_2 experiments were carried out involving their incubation in vacuo. It will be clear from the results in Tables 2 and 3 that oxidation of hemoglobin, induced by 2 low H_2O_2 concentration, proceeds only in the presence of oxygen and not in its absence, but addition of oxygen to the reaction medium after incubation of the reagents in vacuo stimulates the triggering of oxidation (Table 3).

The following conclusions can be drawn from the results given in Figs. 1, 2, and 3 and in Table 1. 1) Addition of H_2O_2 to a solution of HbO_2 initiates a chain reaction, as a result of which a quantity of HbO_2 many times greater than the initial quantity of H_2O_2 is oxidized 2) It follows from the data in Table 1 that the principal oxidizing agent throughout this process is H_2O_2, evidently formed de novo during oxidation of HbO_2. 3) The limiting stage of the reaction is not the stage of interaction between H_2O_2 and hemoglobin, but the process following it, consisting evidently of internal structural changes in the hemoglobin molecule induced by H_2O_2. In that way the data given in Fig. 3 can be explained.

TABLE 3. Oxidation of Rat Hemoglobin after Incubation with H_2O_2 and Exposure to a Vacuum

Serial No.	Exposure of H_2O_2 to vacuum		Concentration of MtHb, %	
			before exposure of H_2O_2 and its addition	After 50 min exposure to H_2O_2 or incubation growth in events 1 and 2
1	—	—	7,1±0,5	7,3±0,4
2	+	—	7,0±0,4	8,4±0,5
3	—	+	7,0±0,4	63,3±2,0
4	+	+	7,1±0,5	66,8±2,8

Legend. Initial H_2O_2 concentration in reaction medium 0.28 μM. Remaining conditions the same as in Table 2, except addition of catalase.

Since H_2O_2, on direct reaction with heme, can oxidize the latter both in the presence and in the absence of oxygen [5, 6], and since MtHb cannot combine with oxygen [12] and, consequently, cannot continue the chain reaction, it can be tentatively suggested on the basis of the data (Tables 2 and 3) that in this case preferential oxidative degradation of the apoenzyme structures responsible for resistance to autooxidation must take place, and as a result, the hemoglobin becomes much less resistant to autooxidation, which also comes about on the addition of oxygen to the reaction medium.

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