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The Oxidation of Haemoglobin

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The literature data on the oxidation of human haemoglobin and the haemoglobin of other vertebrates in the form of complexes with ligands and in the free state are surveyed. The relation between conformational changes in oxyhaemoglobin and the rate of its autoxidation as well as the role of the chemical factors influencing the conformation of the protein, the active centre, and the rate of oxidation of haemoglobin are emphasised.
The bibliography includes 122 references.

CONTENTS

I. Introduction 606
II. The bond between the haem iron and oxygen 606
III. The autoxidation of oxyhaemoglobin 607
IV. The oxidation of haemoglobin by reagents 616
V. The oxidation of haemoglobin under the influence of radiation 620

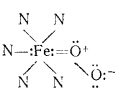
I. INTRODUCTION

Approximately 3% of the circulating haemoglobin (Hb) is oxidised daily in the blood of the adult human being[1]. The reductase systems in the erythrocyte reduce most of the oxidised haemoglobin. However, under these conditions, the blood contains permanently 1–2% of the oxidised form—methaemoglobin (Hb^+) relative to the total amount of the unoxidised and oxidised forms. The small amount of methaemoglobin in the blood plays a definite physiological role: in particular, methaemoglobin actively binds certain anions (CN^-, OCN^-, SCN^-, N_3^-, etc.) which have a high affinity for iron(III) and are capable of binding the iron in cytochromes and of blocking the respiratory chain of the mitochondria[2,3]. An increase of the concentration of methaemoglobin above 1–2% lowers the oxygen transport function of blood. This state, referred to in clinical practice as methaemoglobinemia, belongs to the class of dangerous pathological conditions and requires treatment when it is sufficiently acute. The causes of methaemoglobinemia can be various: hereditary breakdown of the primary globin structure, the breakdown of the functioning of the methaemoglobin reductase systems in the erythrocyte, and the entry into erythrocytes of methaemoglobin-forming substances of endogenic or exogenic origins. In all these cases, uselessly circulating methaemoglobin accumulates in the blood. The understanding of the mechanism of the oxidation of haemoglobin or more precisely of the oxidation of the haem iron of haemoglobin and the conditions promoting or hindering this process ultimately helps physicians to find ways of preventing or reducing methaemoglobinemia. The aim of the present review is to survey the existing literature concerning various pathways leading to the oxidation of haemoglobin with emphasis on the purely chemical aspects of this process.

In discussing the data, preference is given to human oxyhaemoglobin (if the origin of haemoglobin is not otherwise stipulated, human haemoglobin may be assumed). However, when interesting data of chemical importance are available, the haemoglobins of other vertebrate species are also considered. In a number of instances, deoxyhaemoglobin has been considered (in other words oxidation under anaerobic conditions) as well as other ligand derivatives of haemoglobin. The one-stranded haemoprotein myoglobin (Mb)—the oxygen-accumulating system in the muscle which has many properties in common with haemoglobin—is also considered in certain cases in the present review. Anomalous haemoglobins have not been dealt with because their wide variety and in many instances their unusual properties require their consideration in a special review. The fundamental ideas concerning the structure and functions of haemoglobin are also not described because such data have been frequently dealt with in various reviews, monographs, and textbooks. Sufficiently complete information about these questions in the Russian language can be found, in particular, in the review of Sharonov and Sharonova[4].

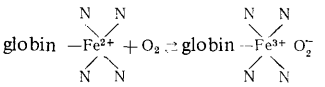
II. THE BOND BETWEEN THE HAEM IRON AND OXYGEN

Oxyhaemoglobin (HbO_2) is a diamagnetic complex of haemoglobin with molecular oxygen in which the haem iron formally retains the +2 oxidation state. The diamagnetism and geometry of this complex still constitute a topic which is attracting the attention of investigators. Among many of the models proposed, those of Pauling[5] and Weiss[6] have been most widely adopted. Pauling assumes that the bond between Fe(II) and O_2 is formed with participation of two *d* electrons of Fe(II) and two *p* electrons of the positively charged oxygen atom in the diamagnetic dipole $^+O-O^-$. The remaining four *d* electrons of the iron are located in its two t_{2g} orbitals, iron also being diamagnetic under these conditions. The structure of Pauling's complex can be represented as follows:



where N denotes the four pyrrole porphyrin atoms and one imidazole nitrogen atom of the histidine protein. The use of the isotope hybrid $^{16}O^{18}O$ in a complex with haemoglobin in the study of Raman spectra[17] has confirmed Pauling's model in which the Fe–O–O group forms an angle of 120°.

Weiss[6] assumes that, when the haem iron interacts with molecular oxygen, an electron is transferred from iron to oxygen:



The single unpaired electron of the iron atom in the $(Fe)^5_{1/2}$ state is then paired with the lone electron of the superoxide radical-anion $\dot{O}_2^-$ (henceforth designated simply by O_2^-) imparting diamagnetic properties to the complex. In support of his model, Weiss points out the known similarity

of the absorption spectra of HbO_2 and alkaline methaemoglobin HbOH. The structure of the complex of Hb with O_2 in which the charges are separated was subsequently supported by means of various methods[8–14]. It has been shown[15] that the length of the O–O bond and the frequency of its stretching vibrations in free O_2^- are the same as the analogous parameters of the O_2 ligand in HbO_2. The Raman spectra of HbO_2 and HbCO at 4 °C in the range 200 to 1700 cm^{-1}, obtained on excitation with a laser at a wavelength of 441.6 nm, agreed with the Raman spectra of the low-spin iron(III) complexes in Hb^+.[12] This justified the claim that the iron in HbCO, like that in HbO_2, is in the +3 oxidation state and is bound to the CO^- anion. However, within the framework of Pauling's model, a partial shift of electron density from iron to oxygen is also permissible. Calculations by various workers yielded different negative charges on oxygen: from 0.1 (Olafson and Goddard[16]) to −0.56 (Kirshner and Loew[17]). The calculated temperature dependence of the quadrupole splitting ΔE_Q in the Mössbauer spectrum of HbO_2 constitutes a satisfactory confirmation of Pauling's model[18]. The concept of the decisive role of the rotation of the O_2 bound to iron was used in the calculations. In the chosen model at a low temperature HbO_2 may exist in one of four potential wells of identical depth due to the interaction of the π^* orbitals of O_2 with the d_{xz} and d_{yz} orbitals of iron and the p_z orbitals of the four nitrogen atoms of the porphyrin. According to the calculation, the height of the barriers between the potential wells is 200 cm^{-1}. The calculated temperature variation of ΔE_Q, determined by the rotation of O_2, agrees exactly with the experimental value within the framework of Pauling's model.

An attractive model of the Fe–O_2 bond in HbO_2, having the electronic configuration of ozone, has also been proposed[19]. The formation of the O_2–O bond in ozone takes place without a marked influence on the O–O bond in the initial O_2 molecule. The singlet ground state of O_3 is stabilised by a three-centre four-electron π-bond. The deviation of the angle between the bonds in O–O–O from 90° (117°) can be explained mainly by the repulsion by the doubly occupied p orbitals of the extreme atoms. The following requirements must be met by Fe(II) for the formation of an analogous bond between the latter and O_2: (1) the presence of the d_z orbital with one electron for the formation of a σ-bond with the analogous p_z orbital of O_2; (2) the presence of the d_{xz} orbital with one electron for the formation of a bond with the π-electron system of O_2; (3) the absence of electrons from the d_{xy} orbital, since the latter is strongly antibonding in relation to the nitrogen atoms of the porphyrin (Fe–O_2 is located in the yz plane passing between the two nitrogen atoms of the porphyrin); (4) the location of the four remaining electrons in the $d_{x^2-y^2}$ and d_{yz} orbitals of iron. Finally the electronic configuration of iron in the proposed model of HbO_2 can be expressed as follows:

$$(d_{x^2-y^2})^2\,(d_{yz})^2\,(d_{xz})^1\,(d_{z^2})^1 \quad \text{or} \quad (t_{2g})^5\,(e_g)^1$$

Ab initio calculation for this model leads to a fully unpolarised Fe–O_2 system.

Thus the survey of the literature on the Fe–O_2 bond in HbO_2 leads to the conclusion that there exist sufficiently well-founded data in support of both ionic and covalent models. Since the main haemoglobin–oxygen dissociation process under normal conditions involves the elimination of the neutral O_2 molecule [Eqn.(1)], i.e.

$$HbO_2 \rightleftarrows Hb + O_2\,, \qquad (1)$$

$$HbO_2 \rightleftarrows Hb^+ + O_2^-\,, \qquad (2)$$

but at the same time there is always at least slight autoxidation of HbO_2 which can be formally described by Eqn.(2), it is reasonable to adopt the covalent bond between O_2 and Hb as the most probable, in conformity with Pauling's model or another similar model, where there is a partial transfer of electron density from iron to oxygen. In other words, it is logical to assume that under normal conditions the contribution of the $Fe^{3+}O_2^-$ structure to the bond between the haem iron and O_2 is fully realistic. However, the concept of a non-polar but effectively polarisable Fe–O bond, in which a negative charge is readily transferred to oxygen under conditions promoting the oxidation of the haem iron is also permissible.

It is likewise essential to note that, if there is an integral negative charge on the oxygen of HbO_2, this does not imply that the iron is in the +3 oxidation state, since the porphyrin system has a considerable capacity for compensating the electron deficiency at the central metal ion. It is difficult to infer the true oxidation state of iron in HbO_2 on the basis of all the available data. Therefore, designations such as Fe^{2+} and Fe^{3+} are in this instance extremely arbitrary and are more in the nature of descriptive symbols[20].

III. THE AUTOXIDATION OF OXYHAEMOGLOBIN

In conformity with the Eqn.(2) presented in the preceding Section, the simplest pathway leading to the oxidation of the haem iron is the dissociation of HbO_2 with the heterolytic rupture of the Fe–O bond. If the medium contains no oxidants other than oxygen molecules under these conditions, then such HbO_2 oxidation is referred to as autoxidation. The autoxidation can be facilitated by agents capable of interacting with O_2 and HbO_2 (reductants of all kinds) or of abstracting O_2^- (protons). Depending on the pH of the medium, the mechanism of the autoxidation of HbO_2 can vary significantly. It is therefore useful to consider this process separately in neutral, acid, and alkaline media.

1. The Autoxidation of HbO_2 in a Neutral Medium

It is known that HbO_2 is gradually modified on storage in neutral aqueous solutions and that one of the main characteristics of this process is the oxidation of HbO_2 to methaemoglobin. However, the low rate of oxidation of HbO_2 at temperatures $\leq$40 °C, where the thermal denaturation of the protein promoting the autoxidation does not play a significant role, hinders the systematic study of the autoxidation of HbO_2 in neutral media. It has been noted[20] that, in the autoxidation of HbO_2 in a neutral buffer, the maximum on the Soret band shifts from 412 to 405 nm, the intensity of the 541 and 575 nm band falls, and a band at 500 nm, characteristic of Hb^+, appears. It has been noted further that the oxidation of HbO_2 is accompanied by its degradation: the band at 280 nm is smoothed out. All these changes are accelerated in a more dilute HbO_2 solution (1.3×10^{-5} M) in which part of the HbO_2 is dissociated into dimers. It has been suggested[20] that the autoxidation of HbO_2 is accelerated by the hydrogen peroxide formed in the reaction, which has been confirmed by special experiments with added catalase, capable of decomposing H_2O_2, which inhibited the autoxidation of HbO_2.

The study of the kinetics of the autoxidation of HbO_2 in 0.1 M phosphate buffer at pH 7.2 showed that the reaction consists of two stages in which the rate constants for the individual stages are 0.032 h^{-1} (degree of reaction ~50%) and 0.0037 h^{-1}. Measurements were also made for the kinetics of the autoxidation of isolated chains under

analogous conditions at 25 °C. The rate constants for the autoxidation of the α- and β-chains proved to be 0.025 and 0.009 h^{-1}, which served as a basis for the assumption that the α- and β-subunits of HbO_2 are oxidised preferentially in the rapid and slow stages respectively. The latter was confirmed by the separation into the individual chains of ~30% oxidised HbO_2 after its treatment with CO and KCN: the isolated α-chains contained 53% of the cyanomet-form, while the β-chains contained only 5% of this form. The different tendencies of the α- and β-subunits of HbO_2 towards autoxidation enabled the authors to suggest that the transition $\alpha_2\beta_2 \rightarrow \alpha_2^+\beta_2^+$ proceeds via the intermediate semi-oxidised form $\alpha_2^+\beta_2$. The reduction of the partial pressure in the system from 57 to 40.5 mmHg increased the rate of autoxidation of mainly the β-chains.

The influence of the Cl^-, F^-, and N_3^- anions on the rate of autoxidation of HbO_2 has been studied at pH 7.4 and 37 °C.[22] The authors suggest that the mechanism of the autoxidation involves in this case the nucleophilic substitution of O_2^- by the corresponding anion. In contrast to the data of Shtamm et al.[20], single-stage oxidation kinetics were observed in this case. At a concentration of the anions of 0.1 M and an ionic strength of 0.5 (in the presence of added sulphate), the rate constants in the presence of Cl^-, F^-, and N_3^- were respectively 0.0229, 0.0348, and 0.175 litre $mole^{-1}$ h^{-1}. At the same time the E_{act} were found to be 32 kcal $mole^{-1}$ for the chloride anion and 26.8 kcal $mole^{-1}$ for the azide anion. Analogous studies by the same workers were carried out in the pH range from 5 to 9.3.[23] The activity sequence was established for a wider range of anions: $N_3^- > SCN^- > OCN^- > F^- > Cl^- > Br^-$. A mechanism was proposed in which the nucleophilic substitution by the superoxide anion by other anions is promoted by the attack of the proton on the oxygen bound to the iron. The low rate of the process compared with the rate of substitution at the haem iron of ligands such as N_3^-, OCN^-, and F^- is explained by the relatively low polarity of the Fe–O_2 bond and the necessity for additional energy to achieve its polarisation. The authors explained the decrease of the rate of autoxidation on passing from an acid to a neutral medium by the decrease of the concentration of the protons interacting with FeO_2.

The mechanism proposed[22,23] for the nucleophilic substitution of O_2^- by other anions is fairly convincing only for the azide anion, whose effect is greatly superior to those of the other anions. The virtual absence of this effect for HbCO (in this case nucleophilic substitution would have involved the unlikely elimination of CO^-) constitutes confirmation of this mechanism for the azide. The linear dependence of the pseudo-first order rate constant on the azide concentration is quoted as another piece of evidence. The absence of a similar linearity for the chloride anion throws doubt on the mechanism of the nucleophilic substitution of O_2^- by the chloride anion as well as other halide anions. According to the above data[22,23], the activity of the halide anions increases from bromide to fluoride in order of decreasing nucleophilicity of these anions in an aqueous medium (contrary to the authors' claim). However, the main objection is the possibility of non-specific and specific interactions of the anions with the protein, in particular in the region of the fixation of DPG, as a result of which the conformational equilibrium $R \rightleftharpoons T$ for HbO_2 is displaced to a greater or lesser extent towards the T-conformation, which can significantly increase the tendency of HbO_2 towards autoxidation. The influence of anions on HbO_2 has been discussed in detail[24].

Whereas the possibility of the nucleophilic substitution of the superoxide anion by salt anions as the sole pathway leading to the autoxidation of HbO_2 in the presence of salts is doubtful, the formation of O_2^- in the reaction medium in the autoxidation of HbO_2 has been quite reliably established. In particular, it has been observed that in the autoxidation of HbO_2 [25] the rate of reaction is inhibited to one-third by catalase. On the other hand, superoxide dismutase, which disproportionates O_2^-, inhibits the autoxidation only slightly. This has led to the conclusion that the O_2^- formed in the system is rapidly converted into H_2O_2, which is actively involved in the oxidation of HbO_2, ensuring its conversion into the oxidised form to the extent of one-third. An analogous effect has been observed also in the autoxidation of human HbO_2.[26]. In this case the reaction was carried out in 0.067 M phosphate buffer at pH 7.4 with added EDTA (to bind traces of Cu^{2+}) at 37 °C. A mechanism involving the following stages has been proposed:

$$\mathrm{Fe(II)\,O_2 \rightarrow Fe(III) + O_2^-},$$
$$\mathrm{Fe(II)\,O_2 + O_2^- + 2H^+ \rightarrow Fe(III) + O_2 + H_2O_2},$$
$$\underline{\mathrm{2Fe(II)\,O_2 + H_2O_2 + 2H^+ \rightarrow 2Fe(III) + 2O_2 + 2H_2O}},$$
$$\mathrm{4Fe(II)\,O_2 + 4H^+ \rightarrow 4Fe(III) + 3O_2 + 2H_2O}.$$

It follows from the above scheme that O_2^- participates actively in the autoxidation of HbO_2. This has been confirmed by the results of another study[27], whose authors suggest that O_2^- gives up one electron to the oxygen in HbO_2, after which the partially reduced oxygen [the superoxide anion bound to Fe(II)] abstracts a second electron from Fe(II) and binds two protons to form H_2O_2. In the native erythrocyte this process does not apparently play an appreciable role, since the concentration of superoxide dismutase in the native erythrocyte is ten times higher, according to the authors' estimate[27], than the concentrations sufficient for the complete suppression of the oxidative effect of O_2^-. The direct involvement of O_2^- in the oxidation of HbO_2 has also been confirmed for a system where O_2^- was generated by the oxidation of xanthine catalysed by xanthine oxidase[28]. The following reactions are postulated in this instance:

$$\mathrm{2H^+ + O_2^- + Fe(II)\,O_2 \rightarrow Fe(III) + H_2O_2 + O_2},$$
$$\mathrm{O_2^- + Fe(III) \rightarrow Fe(II)\,O_2},$$
$$\mathrm{2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2}.$$

It has been established that in a neutral medium the first of the above reactions is suppressed by the third after the attainment of an O_2^- concentration $>10^{-5}$ M. In a study with isolated chains[21] a higher rate of the autoxidation of the α-chains (0.025 h^{-1} at 25 °C) compared with the β-chains (0.009 h^{-1}) was observed. It was suggested that the valence hybrids $\alpha_2^+\beta_2$ are formed as intermediates in the autoxidation of HbO_2. This implies that, for example, in the observed 20% oxidation of HbO_2, 40% of the HbO_2 molecules are actually involved in the oxidation process. According to Tomoda et al.[29], in the autoxidation in 0.01 M phosphate buffer at pH 6.6–7.8 the rate constants for the formation of $\alpha_2^+\beta_2$ and $\alpha_2\beta_2^+$ are respectively 2.2×10^{-2} h^{-1} and 1.88×10^{-2} h^{-1}. The addition of inositol hexaphosphate (IHP) accelerated the oxidation of both subunits approximately by a factor of two in this instance.

The formation of valence hybrids in the autoxidation of haemoglobin can have serious physiological consequences. Analytic isoelectrofocussing in a polyacrylamide gel of the haemolysates of the blood of patients suffering from methaemoglobinemia with a more than 20% Hb^+ content revealed the presence of three fractions: HbO_2, Hb^+, and partly oxidised Hb.[30] The amount of the last fraction was 35–50% of the total amount of the haemoprotein. Thus the determination of the oxidised haemoglobin by the generally accepted Drabkin method yields the concentration of the oxidised haem, but not the amount of the haemoglobin tetramers involved in the oxidation. The increased affinity for oxygen and the almost total absence of cooperative

behaviour are characteristic of the valence hybrids (analogous data have also been obtained in another study[31]). The last two factors significantly impair the oxygen transporting capacity of the blood in patients suffering from methaemoglobinemia.

There are data[32] for the autoxidation of HbO_2 in the blood plasma at pH 7.5 and 37 °C. The results of the study of Mansouri and Winterhalter[21] have been confirmed: the decrease of the oxygen partial pressure in the blood plasma from 126 to 23 mmHg increased severalfold the rate of autoxidation of HbO_2.

The study using the HbO_2 from the frog *Rana esculenta*[33], chosen because of its high lability with respect to autoxidation, established that the rate of oxidation of this oxyhaemoglobin in 0.01 M phosphate buffer at pH 7.0 is significantly influenced by the heavy metal ions present in the buffer. The addition of EDTA greatly decreased the rate of autoxidation in this instance. In the absence of heavy metal ions, 7% of HbO_2 was found to be oxidised after 1 h at 2 °C and at an initial HbO_2 concentration of 1.5×10^{-5} M, whereas in the presence of added 10^{-4} M metals, the following amounts of oxidised HbO_2 were found: 28% for Cu, 16% for Zn, 5% for Sn, 11% for Fe, 14% for Cr, 18% for Mn, 16% for Hg, 11% for Pb, 17% for Co, 13% for Cd, and 20% for Ni. In particular, it was established that copper forms complexes with the imidazole groups of histidine and causes the denaturation of the protein, which is reversible in the presence of EDTA. The catalytic effect of heavy metals was also noted in the autoxidation of sperm whale or ox oxymyoglobin (MbO_2) in various buffers[34]. An equimolecular amount of Cu(II) accelerated in this case the oxidation by a factor of 25. The accelerating effect of Cu(II) on the autoxidation of the sperm whale MbO_2 at pH 5.0 [35] is explained by the binding of Cu(II) by the GH1 histidine, which interacts with haem and accelerates the delocalisation of the valence electron of the iron. According to the authors, this electron may fall into the potential well of the acceptor (the O_2 in the medium or Cu^{2+}) when the latter forms a complex in the region of His GH1.

It has been noted[36] that at pH 7 (0.05 M tris buffer) the addition of $ZnCl_2$, $PdCl_2$, and $CdCl_2$ did not entail spectroscopic changes in the HbO_2 solution, whereas the addition of $FeCl_2$, $FeCl_3$, $CuCl_2$, and $HgCl_2$ did lead to spectroscopic changes and the formation of a precipitate. It is remarkable that the presence of EDTA, which suppresses the effect of Cu(II), does not interfere with the oxidative activity of Fe(II) and Fe(III).

Apart from the study[35] quoted above, there exists a fairly extensive literature on the autoxidation of MbO_2. The exact determination of the amount of free O_2 evolved on formation of Mb^+ is important for the understanding of the mechanism of the autoxidation of MbO_2.[37] It has been shown that only 20% of the O_2 bound in MbO_2 (in the ox and sperm whale) is converted into water, while 75% is evolved in a free state. This suggested the following simple mechanism:

$$MbO_2 \rightleftarrows Mb + O_2 ,$$
$$Mb + H^+ + {}^1/_4 O_2 \rightarrow Mb^+ + {}^1/_2 H_2O .$$

This mechanism was confirmed by the dependence of the rate of autoxidation of MbO_2 on the O_2 concentration and the pH observed by the authors[37]: at pH 7 the autoxidation rate constants were found to be inversely proportional to $[O_2]$ and their logarithms increased linearly as the pH was reduced from 7 to 5. More detailed studies made it possible to elaborate further the above mechanism by including in the latter the intermediate O_2^- and H_2O_2 as oxidants[26]:

$$MbO_2 \rightarrow Mb^+ + O_2^- ,$$
$$MbO_2 + O_2^- + 2H^+ \rightarrow Mb^+ + O_2 + H_2O_2 ,$$
$$2MbO_2 + H_2O_2 + 2H^+ \rightarrow Mb^+ + 2O_2 + 2H_2O .$$

An unusual point of view concerning the possible mechanism of the autoxidation of HbO_2 has been put forward by Banerjee and Stetzkowski[38], who induced the autoxidation of HbO_2 by adding high-molecular-weight polypeptides obtained by the decomposition of horse heart Mb. In this case the transfer of haem from the haem pocket to the peptide was observed with formation of haemochrome. Peptides which readily bind ferrohaem, while the binding of ferrihaem is poor, are the most effective in the autoxidation of HbO_2. The following chain of transformations takes place in this case: the emergence of ferrohaem from the haem pocket, the binding of ferrohaem to the new ligand, the oxidation of ferrohaem, and the return of ferrihaem to the haem pocket. The process proceeds at the maximum rate when the haemoglobin is half-saturated by oxygen and is suppressed when its saturation is complete. Thus the autoxidation of HbO_2 in a neutral medium can be due to the oxidation of partly dissociated deoxyferrohaems derived from Hb in equilibrium with HbO_2. The initial ideas about the mechanism of the autoxidation of HbO_2 have been described in a monograph by Blyumenfel'd[39], who investigated the transfer of haem from haemoglobin to gelatin (the haem transfer reaction). However, in the absence of external ligands promoting the emergence of haem from the haem pocket, the haem dissociation mechanism of autoxidation gives rise to certain doubts bearing in mind that the α-subunits in HbO_2 are oxidised more readily than the β-subunits, while the bond between haem and the globin is stronger in the former than in the latter[21].

An approach to the understanding of the intimate mechanism of the autoxidation of HbO_2 in a neutral medium can be achieved by comparing this process with the autoxidation of protein-free iron(II) complexes. The principal possible mechanisms have been discussed in a review[40]. The simple bimolecular interaction

$$Fe(II) + O_2 \rightarrow Fe(III) + O_2^-$$

is an endothermic process and can hardly serve as a model of the main pathway in the autoxidation of HbO_2. The following scheme is more likely on energy grounds:

$$Fe(II) + O_2 \rightleftarrows Fe(II)O_2 ,$$
$$Fe(II)O_2 + Fe(II) \rightleftarrows Fe(II)O_2Fe(II) ,$$
$$Fe(II)O_2Fe(II) \rightarrow 2Fe(III) + O_2^{2-} \text{ etc.}$$

However, whereas this scheme is suitable for the porphyrin complexes of iron(II), in the presence of a protein environment the formation of a dimer is improbable. Finally, the process involving the transfer of an electron from the medium to the oxygen bound to iron(II) may play a definite role. This can be represented in the following scheme:

$$Fe(II)O_2 + H \rightarrow Fe(II)O_2H ,$$
$$Fe(II)O_2H \rightarrow Fe(III) + HOO^- .$$

However, this process too is relatively ineffective for HbO_2, since the O_2 in the haem pocket is protected from reductants by the distal histidine. There exists the view that the high oxidation-reduction potential of haemoglobin (+0.2 V at pH 7.0) is in fact largely due to this histidine[41].

2. The Autoxidation of HbO_2 in an Acid Medium

In a neutral HbO_2 solution there is a tetramer–dimer equilibrium [Eqn.(3)] which is largely displaced towards the tetrameric form of HbO_2 ($\alpha_2\beta_2$):

$$\alpha_2\beta_2 \rightleftarrows 2\alpha\beta \qquad (3)$$

A deviation of the pH of the solution from neutrality shifts the equilibrium towards the dimers. Even in a weakly acid medium (pH < 6) the concentration of the dimers is appreciable[42]. At pH < 4 considerable structural changes take place and are normalised by the prototropic groups masked in the protein. These include 18 of the 38 imidazole groups present in haemoglobin[43]. Dilatometric studies[42] have shown that at relatively high pH in the acid medium the volume of HbO_2 is somewhat increased, which is characteristic of globular proteins capable of being protonated. However, the protonation of HbO_2, starting from pH 4.5, results in a sharp decrease of the volume, which the authors explain by the intensified dissociation of HbO_2 into dimers.

The structural changes in carboxyhaemoglobin[45], whose conformation and properties are very close to those of HbO_2, have been investigated in detail in the pH range 3.3–1.8. It was noted that at pH > 3 the protein is uncoiled, while the haem remains bound to the native sections of the protein, although its environment is altered. At pH < 3, the structural changes in the protein are more significant: the uncoiling of the protein is accompanied by a considerable decrease of absorption in the region of the Soret band (there is a change in the character of the binding of haem) and also by the disappearance of the anomalous dispersion of the optical rotation and a change in absorption at 269 nm, both associated with the uncoiling of the protein globule. At pH < 2.1 almost all the haem is dissociated. In this case, the kinetics of the spectroscopic changes are independent of pH. It has been noted[46] that the absorption spectrum of the product of the denaturation of HbCO at pH 1.95 depends on the denaturation temperature (from 0° to 25 °C). The authors explain this by the splitting off of haem from the denatured product and its addition to another site in the protein, whose position depends on the process temperature. When the denaturation is carried out at pH 3.2, this type of temperature dependence of the process is not observed, which indicates the stability of the native bond between haem and globin at this pH.

The autoxidation of HbO_2 at a concentration of $\sim 3 \times 10^{-5}$ M has been investigated[47,48] in an acid medium at pH from 5.6 to 4.0 in the presence of various amounts of alkali metal salts. In all cases 0.1 M sodium acetate buffer solution was employed. The reaction rate constants, determined spectrophotometrically (at 576 nm), were mainly satisfactorily calculated in terms of a first-order process up to a considerable extent of reaction. The decrease of pH from 5.6 to 4.0 was accompanied by a linear increase of the logarithm of the rate constant. This justifies the attribution of the change in the activity of the protons of the medium in the above pH range to the same linearly varying factor. The observed linearity indicates a qualitative constancy of this factor as the activity of the proton is increased down to pH 4.0. This factor may be the conformational changes in the protein of HbO_2, which increases the lability of HbO_2 with respect to autoxidation. At the same time, these changes increase the degree of dissociation of HbO_2 into dimers and monomers. In the general case the dissociative-associative equilibrium for HbO_2 in various aqueous media is expressed by Eqn.(4), which supplements Eqn.(3), given above, by allowing for the possibility of the formation of monomers in the system:

$$\alpha_2\beta_2 \rightleftarrows 2\alpha\beta \rightleftarrows 2\alpha + 2\beta \qquad (4)$$

Close to physiological conditions, reliable data concerning any kind of appreciable dissociation of haemoglobin into monomers are lacking, but monomers are detected when the deviation from neutral pH is considerable[49]. On the other hand, it is known that in a neutral medium chlorides increase the tendency of the monomeric subunits towards self-association[50,51] and the formation of the $\alpha\beta$ dimers[52]. Neglecting the possible difference between the rates of autoxidation of the α- and β-subunits, one can formulate a more general expression for the rate of oxidation of HbO_2:

$$\omega = -\frac{d[HbO_2]}{dt} = k_{obs}[HbO_2] = k_4[\alpha_2\beta_2] + k_2[\alpha\beta] + k_1[\alpha + \beta] , \qquad (5)$$

where k_4, k_2, and k_1 are the rate constants for the autoxidation of the tetramers, dimers, and monomers respectively and $[\alpha_2\beta_2]$, $[\alpha\beta]$, and $[\alpha + \beta]$ are the concentrations of the tetramers, dimers, and monomers respectively in terms of haem.

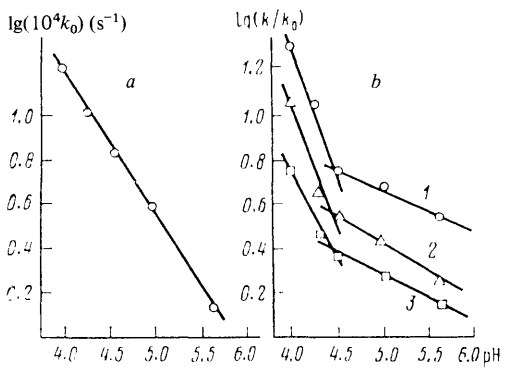


Figure 1. The pH dependence of the logarithm of the rate constant k_0 for the autoxidation of HbO_2 in the absence of chlorides (*a*) and the logarithms of the relative rates in the presence of KCl (*b*); $[HbO_2]_0 = 3 \times 10^{-5}$ M, 40 °C:[48] *1*) 0.333 M; *2*) 0.166 M; *3*) 0.083 M.

While the dependence of the rate of autoxidation of HbO_2 on the pH in the pH range 5.6–4.0 has no characteristic points[48], in the presence of alkali metal chlorides at concentrations up to 0.33 M, the pH dependen e of the logarithm of the rate constant has a sharp break at pH 4.5 (Fig.1). Presumably, there is a break in the curve for the pH dependence of the degree of tetramer–dimer dissociation at this pH in the presence of chlorides. The significant role of the chloride ion can be explained in this instance in the following manner. In the absence or at a low concentration of anions, the gradual increase of the activity of protons increases the number of protonated prototropic groups mainly in the imidazole and carboxylate residues. The accumulation of positive charges in the region of the α^1–β^2 contact increases the Coulombic repulsion of the dimers and gradually shifts the tetramer–dimer equilibrium to the right. In the presence of an electrolyte, the ionic strength of the medium at relatively high pH in the acid region intensifies the hydrophobic interactions between the dimers in the tetramers and to a certain extent prevents the dissociation of the latter (entropy factor). However, after the attainment of a certain level of protonation of the protein (tentatively in the region of pH 4.5), the region of the α^1–β^2 contact becomes readily accessible to the salt anions

as a result of the Coulombic repulsion of the dimers, the anions being directed to the positive charges on the protein mainly without loss of the hydration shell. Furthermore, as the protein is protonated its hydrophobic character diminishes. As a result of all these features, the role of the salt as a factor intensifying the hydrophobic interactions in the protein diminishes in a certain pH range, and the hydrated anions of the medium actually acquire the ability to enter the region of the contact between the $\alpha^1\beta^1$ dimers, destabilising the tetrameric structure. The simultaneous operation of these factors is apparently actually responsible for the appearance of a distinct break in the dependence of the rate of reaction on the pH in the presence of alkali metal chlorides. The dissociation of the $\alpha^1\beta^1$ dimers into monomers, which is possible at pH < 4.5 sharpens the break at pH 4.5. Speaking of the possible dissociation into monomers, the present author and Chundinova[47] emphasised that it is not obligatory to assume the formation of monomers separated by the medium when the "dissociative" influence of the pH on the autoxidation of HbO_2 is discussed: the cause of the strong effects on the autoxidation of HbO_2 can be also the weakening of the $\alpha^1-\beta^1$ contact as a consequence of the conformational changes in the monomers forming part of the dimers, preceding and determining the dissociation of the latter into monomers.

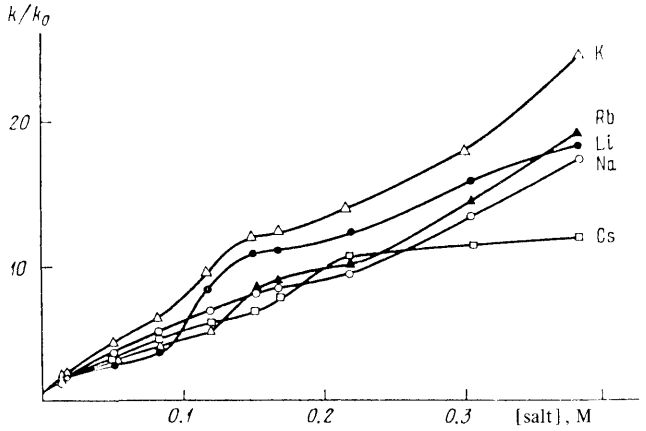


Figure 2. Dependence of the rate of autoxidation of HbO_2 on the concentration of alkali metal chlorides at pH 4.0 and 40 °C; $[HbO_2]_0 \cong 3 \times 10^{-5}$ M (in terms of haem).[47]

It has been established[47,48] that salt cations in an acid medium exert a very distinct influence on the rate of autoxidation of HbO_2. Fig.2 illustrates the curves for the dependence of k/k_0 (where k_0 is the rate constant in the absence of added salt) on the salt concentration for alkali metal chlorides at pH 4.0. The potassium cation is the most active and the caesium cation is the least active at concentrations above 0.25 M. At pH 5.6 the difference between the effects of the individual cations is more appreciable and in this case caesium is also the most passive. At pH 4.0 differences were also demonstrated between the effects of a series of anions. Fig.3 shows that the anions form the following sequence in terms of their accelerating effect on the autoxidation of HbO_2: $F^- < Cl^- < Br^- < I^- < ClO_4^-$.

This sequence is almost identical with Hofmeister's classical lyotropic series. The arrangement of the anions in conformity with the lyotropic series had been observed earlier for the dissociation of HbCO into dimers in a neutral medium under the influence of different sodium and potassium salts[54]. The closeness of the dissociation series to the series of anions in terms of their accelerating effect on the autoxidation of HbO_2 confirms the hypothesis[47,48] that there is a definite similarity between the mechanisms of the processes leading to the dissociation of HbO_2 into dimers and monomers and to its autoxidation.

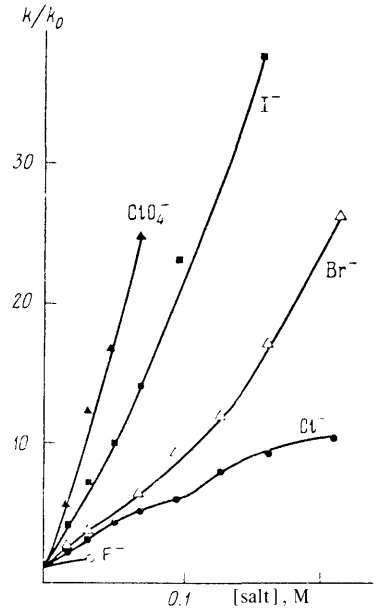


Figure 3. The influence of the sodium salt concentrations on the autoxidation of HbO_2 at pH 4.0 and 40 °C; $[HbO_2]_0 \cong 3 \times 10^{-5}$ M, $k_0 = (11.9-15.6) \times 10^{-4}$ s^{-1}.[47]

The influence of the nature and concentration of a series of carboxylate buffer anions on the autoxidation of HbO_2 has been investigated[55]. It has been observed that at pH 5.6, where HbO_2 is mainly tetrameric, and at pH 4.0, where HbO_2 exists preferentially in the form of dimers, the character of the influence of different carboxylate ions is significantly different. Fig.4 presents a plot of the logarithms of the rate constants for the autoxidation of HbO_2 at 40 °C against the molarity of the buffer. At pH 5.6 carboxylates can be clearly divided into three groups: alkanoates, chloroacetate (in the presence of which the reaction is 4–8 times slower than in the presence of alkanoates), and citrate, which gives rise to a complex concentration dependence. It is seen that small amounts of added alkanoates (up to ~0.2 M) have the most appreciable effect on the autoxidation of HbO_2. The chain length of the alkanoic acid has almost no influence on the effect under these conditions. At pH 4.0 the picture is different. If account is taken of the degree of dissociation of the alkanoic acids at this pH, then the alkanoates give rise to an almost linear dependence of the logarithm of the rate constant in the sequence formate < acetate <

propionate < butyrate, the rate in the presence of the butyrate, when the molarity of the buffers is 0.2 M, exceeding approximately by a factor of 15 the rate in the presence of the acetate. Hence it follows that, under conditions where HbO_2 is almost fully dissociated into dimers, non-polar agents modify extremely effectively the structure of HbO_2, rendering it significantly less resistant to autoxidation, which is not observed for the tetrameric structure of HbO_2 (pH 5.6). Consequently, the tetrameric HbO_2, whose outer surface is mainly hydrophilic, is relatively insensitive to non-polar agents, while the dimeric HbO_2, which has an open $\alpha^1-\beta^2$ contact surface with large hydrophobic sections, is extremely sensitive to non-polar agents.

Parameters of the Arrhenius equation for the oxidation of the sperm whale MbO_2 in 0.015 M tris–phosphate buffer[57].

pH	$t = 10-20°$			$t = 25-35°$		
	$\Delta G^{\neq}_{20}$	$\Delta H^{\neq}$	$\Delta S^{\neq}$, e.u.	$\Delta G^{\neq}_{29}$	$\Delta H^{\neq}$	$\Delta S^{\neq}$, e.u.
5.0	3.59	6.4	9.6	3.48	20.8	59.1
5.4	3.72	6.8	10.5	3.63	22.0	62.7
5.8	3.66	9.2	18.9	4.05	26.0	74.9
6.0	3.98	11.6	26.0	4.20	29.6	86.7
6.4	4.49	19.2	50.2	4.65	37.2	111.1
6.8	5.33	28.8	80.1	5.06	44.0	132.9

Measurements have been made for the kinetics of the denaturation of horse HbO_2 in the pH range 3.6–2.4 at 0 °C.[56] With decrease of pH in the above range, the first-order rate constant increased exponentially. Comparing this finding with the data of the present author and Chudinova[48], one can assume that, in the absence of an excess of salts, the denaturation and autoxidation processes (stimulated in all probability by the same factors), involving HbO_2 from different species, are proportional to the activity of the protons over a fairly wide pH range in the acid region.

Data on the autoxidation of ox MbO_2 at pH 7.0–5.0 [37] provide some justification for extending this pH range to 7.0. At the same time, it must be emphasised yet again that the "dissociative" mechanism of the autoxidation of HbO_2 formulated above presupposes conformational changes in the protein preceding the dissociation of the haemoglobin into subunits and not the dissociation itself. The character of these changes is apparently mainly the same as for the one-stranded myoglobin, which is confirmed by the linear dependence of the rate constant for the autoxidation of MbO_2 on the activity of protons[37].

The main parameters for the oxidation of the sperm whale MbO_2 have been determined in the pH range 7–5 in 0.015 M tris–phosphate buffer[57]. A linear dependence of the quantity $\lg [V/(1 - V)]$, where V is the Mb^+ concentration at a specific instant during the initial period of the reaction, on the pH was observed at 35 °C. Bearing in mind that the slope of this relation is two, the authors suggested that the autoxidation of MbO_2 is catalysed by the protonation of two histidine residues. The curve for the temperature variation of the rate of reaction has a break between 20° and 25 °C. The parameters of the Arrhenius equation observed at different pH are listed in the Table. The difference between these parameters for the temperature ranges 10–20° and 25–35 °C provided the authors with the grounds for the claim that there are two mechanisms of the autoxidation of MbO_2. It has been observed that the dependence of $\ln k$ on $\sqrt{I}$ (I is the ionic strength of the solution) at different temperatures is a minimum at $\sqrt{I} \approx 0.2$. The decrease of the rate of autoxidation of MbO_2 with increase of the ionic strength of the solution up to $I = 0.04$ is explained by the authors by the shielding of the positively charged groups of the protein, which are important for the autoxidation of MbO_2.

In conclusion of this Section, one can briefly formulate the principal possible causes of the acceleration of the autoxidation of HbO_2 in acid media. These may be (1) the conformational changes induced by the protonation of the weakly basic centres of the protein accompanied by the tetramer–dimer and dimer–monomer dissociation; (2) the protonation of the distal histidine, which enhances the hydrophilic properties of the haem cavitity, which can

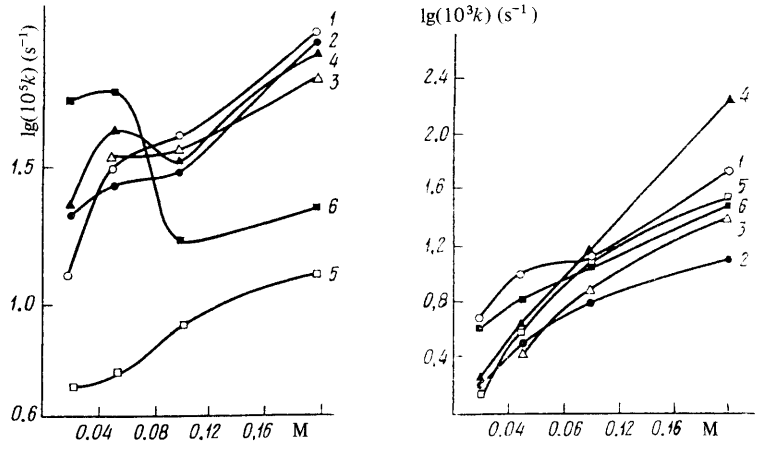


Figure 4. Dependence of $\lg k$ for the autoxidation of HbO_2 on the molarity of the buffer systems at 40 °C and $[HbO_2]_0 \approx 3 \times 10^{-5}$ M: *a*) pH 5.6; *b*) pH 4.0; *1*) formate buffer; *2*) acetate buffer; *3*) propionate buffer; *4*) butyrate buffer; *5*) chloroacetate buffer; *6*) citrate buffer[55].

facilitate the approach of the anions from the external medium to the iron and the emergence of O_2^-; (3) the protonation of the O_2 bound to the iron which promotes the electron transfer from Fe(II) to O_2 with subsequent elimination of HOO$^{\cdot}$.

3. The Autoxidation of HbO_2 (and the Oxidation of HbCO)

The mechanism of the action of alkali on haemoglobin has been most fully investigated by Perutz[58]. The main ideas reduce to the following. The free energy of the spherical polyelectrolyte is proportional to the square of its purely surface charge, as a consequence of which the stability of the polyelectrolyte diminishes on either side of the isoelectric point during the discharge of the cationic or anionic groups. Titration with acid or alkali can ionise the weak acids or bases concealed in the non-polar nucleus. After ionisation, these groups attract the hydration shell, which alters the native structure of the protein and shifts the equilibrium towards the uncoiled form. Three haemoglobins have been compared: human haemoglobin A, foetal haemoglobin (F), and ox haemoglobin. In 0.1 M NaOH at 20 °C, HbCO(A) is denatured in 20 s and HbCO(F) is denatured after 12 min, while the denaturation of the ox HbCO in 0.5 M NaOH has a 3 h half-decomposition period. Furthermore, it is known that all three haemoglobins dissociate into dimers at pH > 10. Hence follows the conclusion that the dissociation into dimers is reversible and does not constitute denaturation in the strict sense of the term. After the attainment of pH 11.6, the HbCO(A) dimers are cleaved and denatured, while the dimers of the two remaining HbCO are denatured only under more severe conditions. It has been suggested that this stage is in fact rate-limiting. Detailed analysis of the aminoacid composition of the three haemoglobins and comparison of the pK_a of various aminoacid residues led Perutz to the following conclusion: the cause of the instability of HbA in alkali is the presence in the latter in the region of the $\alpha^1-\beta^1$ contact of two Cys (G11α and G14β) units and also of Tyr H8β. In water the pK_a of the sulphhydryl groups of cysteine is 9.1–9.5 and that of the tyrosine hydroxyl is 9.5–10.0. Since these groups are strongly masked in haemoglobin, their true pK_a's are higher, but all these groups are undoubtedly ionised at pH > 12. Foetal haemoglobin has only one of the three residues specified (Cys in the γ-chain), while ox haemoglobin contain no such groups at all, which is the cause of its extremely high stability in alkali. Fig.5 illustrates schematically the dissociation of the dimer and the uncoiling of the monomeric chains under the influence of alkali. The author notes in this connection that there is no reliable information showing whether the dissociation into monomers or denaturation is the primary process.

The influence of the pH of the solution on the rate of autoxidation of human HbO_2 in the NaOH–glycine buffer in the pH range 9.0–12.0 at 40 °C has been studied in detail[59]. The observed relation is illustrated in Fig.6, which shows that the rate of autoxidation, which increases monotonically with increase of pH over the entire range investigated, gives rise to a pH dependence consisting of four clearly distinct sections. In the pH range 9–10 the rate constant increases linearly from 0.75 to 1.6×10^{-5} s^{-1}, i.e. approximately by a factor of 2. According to the available data for HbCO,[60] the concentration of the dimers increases approximately by a factor of 2 in the same pH range—from ~12 to ~24%. This agreement suggests that the rate of autoxidation of HbO_2 in the pH range 9–10 is determined mainly by the degree of tetramer–dimer dissociation. In the pH range 10–10.6 the rate constant increases linearly almost by an order of magnitude. Presumably the sharp break in the relation at pH 10.0 is caused not only by the rapid increase of the concentration of dimers (approximately by a factor of 3) but also by the conformational changes in the dimers formed. Next, the curve for the relation exhibits a further two breaks: at pH 10.6 and 11.6. The temperature variations of the rate of autoxidation have been determined for all three pH values and the following activation parameters have been calculated:

pH 10.7 $E_{act} = 60.3$ kcal mol^{-1}, $\Delta S^{\neq} = +116$ e.u., $\Delta G^{\neq} = 25.2$ kcal mol^{-1};
pH 11.45 $E_{act} = 18.7$ kcal mol^{-1}, $\Delta S^{\neq} = +15.7$ e.u., $\Delta G^{\neq} = 21.4$ kcal mol^{-1};
pH 12.0 $E_{act} = 10.9$ kcal mol^{-1}, $\Delta S^{\neq} = -36.7$ e.u., $\Delta G^{\neq} = 19.9$ kcal mol^{-1}.

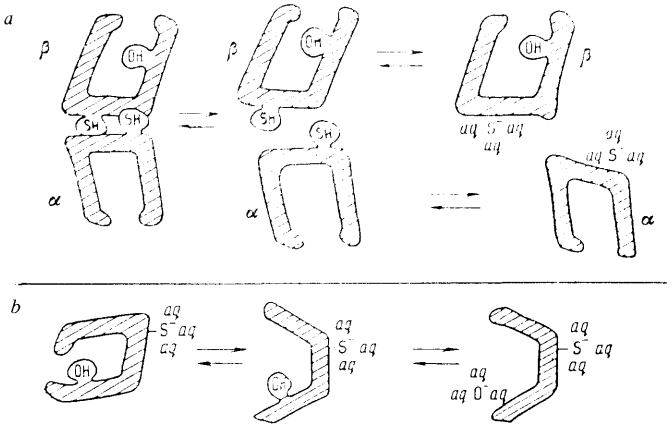


Figure 5. Schematic representation of the alkaline denaturation of haemoglobin according to Perutz[58]: *a*) dissociation of the dimer and ionisation of cysteine; *b*) uncoiling of the monomer and ionisation of tyrosine.

High enthalpies and entropies of activation are characteristic of the denaturation of proteins, in particular at pH 8, E_{act} = 77.3 kcal mol^{-1} and $\Delta S^{\neq} = 153$ e.u. for the thermal denaturation of human HbA.[61] On the other hand, the activation energy for the transition Fe(II) → Fe(III), involving simple iron complexes in the reaction with O_2, is of the order of 12–15 kcal mol^{-1}.[62,63] A negative entropy of activation for the oxidation reaction is also characteristic of all simple complexes. All these factors suggest that at relatively low pH values (9–10.5) the rate of autoxidation of HbO_2 is limited by conformational changes in the protein; on the other hand, with increase of pH their rate increases and the rate-limiting stage becomes the oxidation of iron in the already denatured haemoglobin (possibly in the dissociated haem). An attempt has been made at the interpretation of the kinetic results by comparing them with the absorption spectra in the range 245–320 nm at 10 °C (in order to detect only rapid conformational changes in HbO_2 under the influence of alkali). As was to be expected, an increase of pH from 9 to 10 hardly affects the spectrum. At pH 11, there is an appreciable increase of the absorption in the range 250–260 nm (normalisation of phenylalanine) and a shoulder appears in the range 285–295 nm (tryptophan, tyrosine). At pH 12 and 13 the spectral changes become more distinct. Thus one can assume that at pH 11 appreciable conformational

changes in HbO_2 already occur and these can cause, in particular, the unmasking of the tyrosine and the unreactive cysteine residues, whose ionisation, in conformity with the ideas of Perutz[58] and the present author and his co-workers,[59] leads to accelerated denaturation and autoxidation of HbO_2.

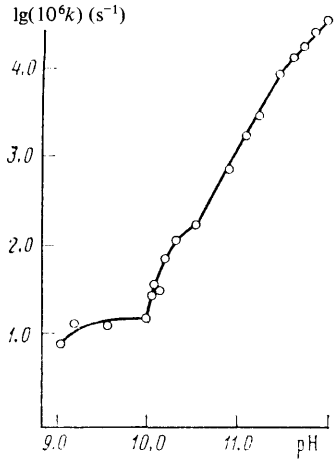


Figure 6. Dependence of $\lg k$ on the pH in the alkaline region for the autoxidation of HbO_2 at 40 °C; $[HbO_2]_0 \cong 3 \times 10^{-5}$ M.[59]

The kinetics of the oxidation of HbCO at pH 11.0–11.9 at 39 °C have been investigated[64] in the absence of molecular oxygen. In contrast to the data obtained in the study by Wallace et al.[23], where HbCO was oxidised at neutral pH in the presence of azide anions more slowly by 6–8 orders of magnitude than HbO_2, the rate of oxidation of HbCO in an alkaline medium proved to be comparable to the rate of autoxidation of HbO_2. Furthermore, the break observed in the dependence of $\lg k$ on pH for HbO_2 [59] was reproduced also for HbCO, although at a somewhat lower pH (11.4). This permitted the conclusion that, in the pH range 11–12, the mechanism of the oxidation of HbO_2 is the same as that of the oxidation of HbCO. In all probability at pH ranging from 11 to 11.4–11.6 the rate of oxidation of both HbO_2 and HbCO is limited by the protein denaturation stage, while at higher pH the rate-limiting stage is the oxidation of the denatured haemoprotein. The activation energy for the oxidation of HbCO at pH 11.65 (15.3 kcal mol^{-1}) is virtually identical with the corresponding value for HbO_2. At pH close to 12 the rate constants for HbO_2 and HbCO are very similar, whence follows the conclusion that in both cases the rate-limiting stage at high pH involves the transformation of the same substrate. Presumably the substrate is deoxyferrohaem. The nature of the direct oxidant of iron in HbCO was not elucidated in the present author's study[64]. Oxidation under aerobic conditions hardly altered the rate constant. In contrast to the first-order oxidation of HbO_2 which proceeds smoothly, the oxidation of HbCO takes place as a two-stage process—the first, faster stage continues up to ~50% degree of reaction.

The autoxidation of HbO_2 has been investigated in the presence of 0.01 M KCN without a buffer (pH ~10.4) with the addition of various amounts of primary normal aliphatic C_1–C_8 alcohols[65]. The comparative catalytic activity of the alcohols varies in this sequence as follows: 1; 2.2; 7.2; 17.5; 47.1; 893; 4010; 7500. The sharp increase of the activity of the alcohol on passing from 1-pentanol to 1-hexanol is noteworthy. This is probably associated with the greater capacity of 1-hexanol and higher-molecular-weight alcohols with a straight chain for insertion via the chain into the internal and mainly hydrophobic regions of haemoglobin.

The greater importance of non-polar effects compared with polar effects was manifested even more distinctly in the oxidation of HbCO in an alkaline buffer (pH 11.35) in the presence of tetra-alkylammonium bromides[66]. Tetramethylammonium bromide at concentrations up to 0.04 M (HbCO concentration 3×10^{-5} M) hardly influenced the rate of oxidation of HbCO. Its tetraethyl homologue showed a significant accelerating effect, while 0.01 M tetrabutylammonium bromide approximately doubled the rate of oxidation of HbCO.

Measurements have been made of the rate of autoxidation of HbO_2 in an alkaline medium in the presence of several stable nitroxy-radicals of the piperidine series[67]. The kinetic data made it possible to estimate the ability of these compounds to interact with HbO_2. The acceleration of the autoxidation of HbO_2 was determined as a measure of the conformational changes in the globin in the presence of different amounts of stable radicals up to the molar ratio radical : haem = 10 : 1. At pH 10.75 it was established that the 2,2,6,6-tetramethylpiperidin-1-yloxy-radical and its 4-oxo and 4-hydroxy-derivatives, which are widely used in the spin probe method, increase the rate of autoxidation of HbO_2 by a factor of 1.5–2 at the maximum concentrations employed. A somewhat greater effect (acceleration by a factor of three) is shown by the 2,2,6,6-tetramethyl-4,4-dinitropiperidin-1-yloxy-radical, which had not been used earlier in practice. Its 4-chloro-4-nitro-analogue proved to be still more active (acceleration by a factor of five). The geminal chloro- and nitro-groups apparently enable this nitroxy-radical to be bound specifically to particular polar centres of the HbO_2 molecule. At lower pH the influence of the nitroxy-radicals on the kinetics of the autoxidation of HbO_2 is less pronounced.

4. The Influence of Sulphydryl Reagents on the Autoxidation of HbO_2 (and the Oxidation of HbCO)

It is known that the blocking of the sulphydryl groups of haemoglobin by organomercury compounds, in particular by *p*-chloromercury benzoate (PCMB) promotes the dissociation of haemoglobin into dimers and monomers, which can be used to prepare isolated haemoglobin subunits[68]. It has been established that in the presence of an excess of PCMB the degree of dissociation of human haemoglobin into subunits depends on the concentration of the added NaCl, the degree of dissociation being a maximum in the presence of 0.1–0.2 M NaCl.[69] At a constant NaCl concentration (0.2 M) in the pH range 5.5–7.5 the degree of dissociation increases with decrease of pH and becomes complete already at pH 6 (at lower pH a precipitate is deposited). At pH 9–11, the dissociation of HbCO is incomplete and its extent decreases with increase of pH. Electrophoretic analysis showed that at low PCMB concentrations isolated subunits do not appear. Small amounts of the subunits are present when the PCMB : Hb ratio is 4 : 1 and a complete separation into subunits is achieved with the ratio 6 : 1. On the basis of these data and comparative data on foetal haemoglobin, the following conclusions have been reached: the reaction of PCMB with reactive SH groups (β-93) leads to the dissociation of HbCO into dimers; the reaction with β-112 SH-groups begins before the completion of the dissociation into dimers

and competes with the reaction of PCMB with the β-93 SH-groups. The dissociation into dimers is completed after both β-SH groups have reacted. The α-104 SH groups react with PCMB only after the completion of the reaction of the latter with the β-112 SH groups. The latter limitation is not obligatory at low pH, for example, at pH 4.7.

It has been noted[70] that, although the β-93 SH groups are not involved directly in cooperative interactions, the position of these groups in the mechanically important region of the protein makes their modification important for the state and functioning of HbO_2. Directly next to the β-93 SH groups on the surface of the $\alpha^1-\beta^2$ contact, there is histidine β-92 (the proximal histidine) and aspartyl β-94—the residue stabilising the deoxy-conformation of the C-end and of the β-chain as a result of the formation of a salt bridge with the His β-146 side chain. This interaction is directly associated with the Bohr effect and involves a cooperative mechanism. There are data showing[71] that the modification of the β-93 SH groups by a number of phenylmercury chloride derivatives increases the affinity of Hb for O_2. An analogous conclusion has been reached also for haemoglobin modified by cystamine and the dimethyl ester of cystine in acid and neutral media[72]. The Bohr effect then diminished and the shape of the oxygen equilibrium curves remained almost unaltered.

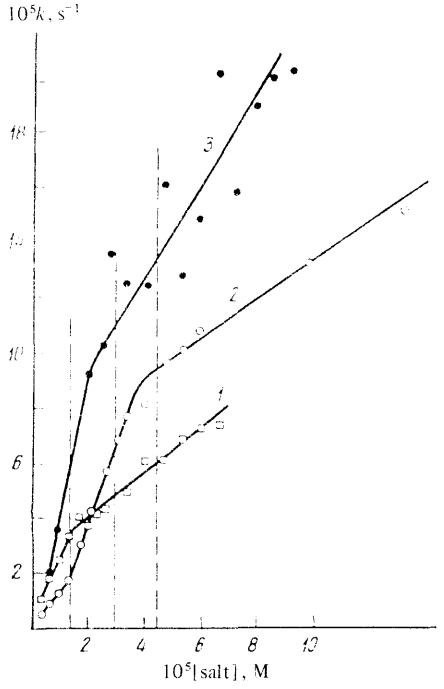


Figure 7. Dependence of the rate constant for the oxidation of HbCO in acetate buffer at pH 4.0, 30 °C, and $[HbCO]_0 = 3 \times 10^{-5}$ M on the concentrations of phenylmercury acetate (curve *1*), benzylmercury acetate (curve *2*), and mercury acetate (curve *3*).[74]

It has been observed[73] that the absorption spectra of Hb at pH 5.5–5.8 with added mercury(II) chloride resemble the spectra in the early stage of the acid denaturation of Hb (at pH 3, the Soret peak is reversely displaced to the region 370–390 nm and suffers an appreciable loss of intensity). Hence follows the conclusion that $HgCl_2$ interacts both with SH groups and with the imidazole residues of the protein.

The rates of oxidation of HbCO at pH 4.0 in the presence of phenylmercury acetate, benzylmercury acetate, methoxycarbonylmercury chloride, and mercury acetate have been measured spectrophotometrically at 30 °C and at a wavelength of 574 nm.[74] Fig.7 presents the curves for the dependence of the rate constant for the oxidation of HbCO on the concentrations of phenylmercury acetate, benzylmercury acetate, and mercury acetate. For the first two substances, an almost linear increase of the constant is observed up to reactant concentrations corresponding to the concentration of the reactive sulphydryl groups. Next, for phenylmercury acetate, the slope of the curve diminishes, while for benzylmercury acetate it actually increases. The results of the study have been interpreted taking into account the almost complete dissociation of HbCO into dimers at pH 4.0.[42] In the presence of benzylmercury acetate, the acceleration of the oxidation of HbCO after the binding of the reactive SH groups is due to the stronger effect of the reaction leading to the dissociation of the dimers into the monomers. The second break in the curve occurs in the region where the reaction of the reagent with the four SH groups of HbCO is completed. Hence one may conclude that the β-122 SH groups interact stoichiometrically and fairly rapidly with benzylmercury acetate. On the other hand, a further increase of the concentration of the latter is accompanied by a slower increase of the rate constant: in all probability the blocking of the β-112 SH groups does not entail the complete dissociation of the dimers and this process is completed only after the binding of the least accessible α-104 SH groups. A similar curve but with more distinct breaks has been obtained also for methoxycarbonylmercury chloride. In the case of phenylmercury acetate the first break in the curve also indicates the stoichiometric binding of the β-93 SH groups. On the other hand, the subsequent decrease of the slope of the curve and the absence of a break in the region corresponding to the four SH groups suggest the absence of the selective interaction of phenylmercury acetate with the β-112 SH groups. Despite the considerable scatter of the results the curve for mercury acetate (Fig.7) makes it possible to assert that, when account is taken of the "bivalence" of this salt, its effect is comparable in magnitude to that of the "univalent" benzylmercury acetate. On passing from the acetate buffer to the phosphate–citrate buffer, the chelating effect of the citrate in relation to the mercury compounds, inhibiting their activity, was observed. The important role of the reaction temperature, which influences the selectivity of the interaction of the mercury reagent with SH groups of different types has been demonstrated in relation to the oxidation of HbCO in the presence of added benzylmercury acetate[75]. Thus, following an increase of the reaction temperature from 30° to 39 °C at pH 4.0, the break characterising the stoichiometric selective binding of the reactive SH groups disappears, while the break in the region corresponding to the four SH groups is retained. An increase of temperature probably unmasks the β-112 SH groups at pH 4.0 and makes them just as accessible as the β-93 SH groups. Furthermore, it has been observed that at 39 °C the decrease of the pH in the range 4.85–4.0 (acetate buffer) also leads to a decrease of the selectivity of the interaction of benzylmercury acetate with SH groups of different types.

In the study quoted above[67], it was shown by the kinetic method described here that the new stable 4-bromo-2,2,6,6-tetramethyl-4-nitropiperidin-1-yloxy-radical is selectively

bound in alkaline media to the reactive SH groups of HbO_2, hardly altering the rate of autoxidation of HbO_2 but inducing a significant acceleration when it is bound to the unreactive SH groups of HbO_2. A procedure can be proposed for the estimation of the degree of binding and the denaturing effect of the new spin labels.

5. The Autoxidation of HbO_2 in the Presence of Reductants

It is known that the autoxidation of HbO_2 can be accelerated in the presence of reductants. Phenols, hydroquinone[76], phenylhydrazine[77,78], and aniline[78] have been most frequently tried as the latter. The role of the reductants in the oxidation of HbO_2 is usually explained[76] by assuming that the reductant transfers an electron bound by the haem iron to the oxygen, which simultaneously receives an electron from the iron and is reduced (after the binding of two protons) to H_2O_2. It is then also assumed that there is a posibility of two main mechanisms. Firstly, the reductant can interact with O_2^- after its formation, i.e. after the electron transfer from iron to oxygen. In this case the acceleration of the autoxidation of HbO_2 is comparatively slight. The other mechanism presupposes the interaction of the reductant with O_2^- in its formation stage, which is in this case rate-limiting. The reductant then accelerates precisely this stage, which results in an appreciably greater increase of the rate of autoxidation.

It has been observed[80] that the autoxidation of HbO_2 is accelerated also by a biological accelerator—glutathione (G). It has been shown that the formation of Hb^+ in its presence is due to hydrogen peroxide. The latter oxidises HbO_2 much more effectively than glutathione at the same concentration. The rate of formation of Hb^+ is proportional to the square of the concentration of glutathione, which made it possible to put forward the following simplified scheme for the process:

$$2G{-}SH + O_2 \rightarrow G{-}S{-}S{-}G + H_2O_2 ,$$
$$Hb \xrightarrow{H_2O_2} Hb^+ .$$

The influence of hydroquinone and catechol was studied in relation to the autoxidation of sheep and ox HbO_2 [76] in 0.05 M phosphate buffer at pH 6.8 with added EDTA at 37 °C. It was established that both dihydric phenols act catalytically, i.e. can be quantitatively isolated after the completion of the oxidation of HbO_2. In the presence of a tenfold excess of hydroquinone the arrest of the reaction was observed in the first ten or more seconds, which could be eliminated by adding a small amount of *p*-benzoquinone. Next it was observed that, in the absence of hydroquinone, benzoquinone reacts stoichiometrically with HbO_2. Thus in the proposed mechanism the benzoquinone formed initially as a result of the oxidation of hydroquinone oxidises HbO_2 with elimination of oxygen. The semiquinone formed oxidises in its turn a second HbO_2 molecule, which regenerates the benzoquinone. It has been established[81] that the oxidation of HbO_2 by benzoquinone in a phosphate buffer at pH 6.8 proceeds as a second-order reaction. There exists the view[82] that the hydroquinone participating in the oxidation of HbO_2 is first incorporated in the ligand environment of haem, after which two-electron reduction of O_2 takes place with the simultaneous oxidation of the haem iron and the exogenic ligand. The formation of O_2^- in the analogous HbO_2 oxidation reaction in the presence of phenylhydrazine has been demonstrated indirectly: by the reduction of ferricytochrome in the presence of HbO_2 and phenylhydrazine and by the oxidation of epinephrine.

There exist also data for the acceleration of the oxidation of haemoglobin in the presence of ascorbic acid[83], imidazole[83], hydroxylamine[81], and different amines and amides[79].

IV. THE OXIDATION OF HAEMOGLOBIN BY REAGENTS

1. The Oxidation of Haemoglobin in the Presence of Copper(II)

As already mentioned above, bivalent copper ions have an accelerating effect on the autoxidation of HbO_2, which greatly exceeds the effect of other metal ions[33]. The natural question arises whether Cu(II) is involved directly in the oxidation of the iron of HbO_2 in the presence of O_2 [Eqn.(6)] or whether it influences the structure of the protein [Eqn.(7)]:

$$Cu(II) \cdots HbO_2 \rightleftarrows Cu(I) \cdots Hb^+ + O_2 , \quad (6)$$
$$Cu(II) \cdots HbO_2 \rightleftarrows Cu(II) \cdots Hb^+ + O_2^- . \quad (7)$$

The mechanism of the action of Cu(II) has been studied in detail[84] using horse haemoglobin. It has been shown that the autoxidation of the purified HbO_2 at the normal pH is due mainly to traces of Cu(II) in solution. The addition of 10^{-4} M EDTA to 7.2×10^{-5} M HbO_2 sharply inhibits the autoxidation reaction. It has been shown by calculation that, in the absence of EDTA, an appreciable acceleration of the autoxidation is ensured by even 1–2 Cu(II) ions per 1000 haem species [the haemolysate contains 1.7 Cu(II) ions per 1000 haems]. The removal of O_2 from the system simplifies the situation: the entire copper is rapidly reduced quantitatively by the haemoglobin, after which the oxidation of Hb no longer takes place (Fig.8). In the presence of O_2, rapid initial oxidation of HbO_2 also takes place, after which comes the stage in which the oxidation is appreciably slower. The author suggests that the rapid oxidation of HbO_2 is caused by the direct transfer of an electron from Fe(II) in HbO_2 to Cu(II). In the slow stage the same process takes place, but the rate of the stage is limited by the regeneration of Cu(II) and Cu(I) in accordance with the equation

$$4Cu(I) + O_2 + 4H^+ \rightarrow 4Cu(II) + 2H_2O .$$

Comparison of the curves in Fig.8 shows that the rate of the rapid HbO_2 oxidation stage is lower than the rate of oxidation of Hb. This is an argument in support of the preliminary dissociation reaction $HbO_2 \rightleftharpoons Hb + O_2$ with conversion of the *R* form of haemoglobin into the *T* form, which, judging from all the evidence, shows a greater tendency towards oxidation by copper. The virtually complete absence of the oxidation of the weakly dissociated HbCO under these conditions constitutes a satisfactory confirmation of this finding. The question arises why Cu(II) oxidises Hb more effectively than other metal ions having a higher oxidation potential. This might be accounted for by the specific binding of Cu(II) to Hb, which facilitates the electron transfer. The equilibrium dialysis in 0.05 M tris buffer at pH 7.2 with the ratio Cu:haem = 1:2 showed that Cu(II) is bound to Hb, but only to 2 of the 4 subunits. This explains naturally why the degree of oxidation of HbO_2 or Hb presented in Fig.8 does not exceed 50%.

The latter conclusion was confirmed in relation to human haemoglobin[85]. It follows from Fig.9 that, when between two and four Cu(II) ions are transferred to the HbO_2 tetramer, there is a sharp jump in the rate of the rapid oxidation stage. On the other hand, this stage is fully inhibited by the blocking of the β-93 SH groups by iodoacetamides. Thus Cu(II) oxidises only the β-chains of human Hb and HbO_2.

This has been confirmed by the observed inertness of Cu(II) with respect to isolated α-chains, while isolated β-chains (the β_4 tetramer) were oxidised very rapidly. Superoxide dismutase and catalase inhibit the slow stage involving the oxidation of HbO_2 but do not affect the rapid stage. This confirms the direct oxidation of HbO_2 by copper in the rapid stage and the catalytic autoxidation of HbO_2 in the slow stage accompanied by the formation of O_2^- and H_2O_2 in the system.

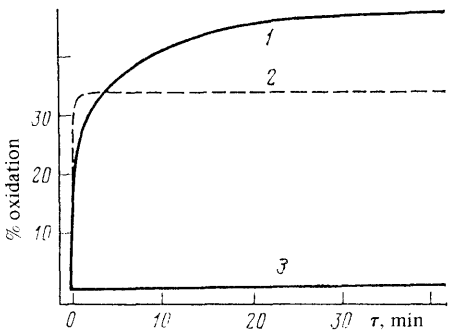


Figure 8. Time variation of the percentage oxidation of 1.42×10^{-4} M solutions of haem derived from horse HbO_2 (curve *1*), Hb (curve *2*), and HbCO (curve *3*) (in the absence of O_2) in a bis–tris buffer (pH 7.0) at 24 °C with added 5×10^{-5} M Cu(II);[84] τ is the time.

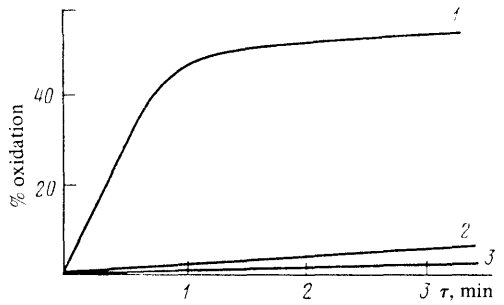
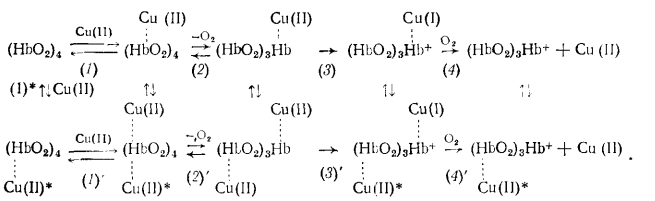


Figure 9. Kinetic curves for the oxidation of HbO_2 by four Cu(II) ions per tetramer (curve *1*), two Cu(II) ions per tetramer (curve *2*), and an excess of Cu(II) in the presence of iodoacetamide (curve *3*);[85] τ is the time.

Dialysis showed that human HbO_2 has four centres for the strong binding of Cu(II). In addition, there are also several (probably two) centres for the weak binding of Cu(II). It has been suggested that the first pair of Cu(II) ions are bound to the β-93 SH groups; the second pair, which ensures the rapid oxidation of HbO_2, is bound to centres located in the vicinity of haem, which ensures the transfer of an electron from Fe(II) to Cu(II) and the formation of haemochrome. The oxidation of the iron in the haem species is followed by the oxidation of the β-93 SH groups with formation of β-dimers containing the disulphide linkage. The presence of these dimers in the reaction product has been observed by gel electrophoresis. The third pair of bound Cu(II) ions are the cause, in the authors' view, of the experimentally observed precipitation of the haemoprotein.

Rifkind et al.[86] failed to confirm the binding of Cu(II) to the β-93 SH groups and also the formation of β-dimers by a procedure involving the modification of HbO_2 by *N*-ethylmaleimide and the comparison of the degrees of binding of Cu(II) to various haemoglobins (human, rabbit, horse, sheep, and bovine haemoglobins). Instead, it was suggested that in the case of human HbO_2 the first two Cu(II) ions are strongly bound to His β-2 or β-116 located on the proximal side in relation to haem and at a fairly long distance from it. At the same time, these residues are located close to the β-93 SH groups and the modification of the latter influences the binding of Cu(II) to His β-2 or β-116. Fig.10 presents for comparison curves for the oxidation of horse and human HbO_2 as a function of the Cu(II) concentration. The indistinct break in the curve for human HbO_2 in the presence of 0.5 of a Cu(II) ion per haem moiety indicates the competitive binding of Cu(II) to the first and second pairs of centres for the binding of Cu(II). A scheme incorporating individual stages in which Cu(II) is bound and HbO_2 oxidised has been proposed:



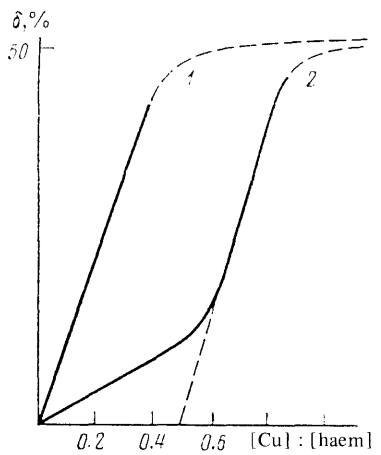


Figure 10. Dependence of the percentage of HbO_2 oxidised in the rapid stage (δ) on the amount of added Cu(II):[86] *1*) horse HbO_2; *2*) human HbO_2.

Stages (1)–(4) obtain in the case of horse HbO_2. For human HbO_2, stages (*1'*)–(*4'*) compete with the above stages. It follows from the scheme that in the latter case HbO_2 without a strongly bound Cu(II) ion can also participate in the haem iron oxidation step (stages marked by an asterisk).

It has been observed[87] that, in contrast to human and horse HbO_2 and Hb, in the sheep haemoglobins Cu(II) rapidly oxidises the haem moieties not only in the β-subunits

but also in the α-subunits. The experiments were performed at pH 6.9 in the absence of buffer in order to prevent the formation of Cu(II) complexes which might influence the mechanism of the action of this ion. The activation energies have been calculated as 53 kJ mol^{-1} for HbO_2 and 113 kJ mol^{-1} for Hb.

We shall consider in greater detail the study already mentioned, where the mechanism of the oxidation of sperm whale myoglobin by copper(II) was investigated[34]. The initial rate of oxidation of MbO_2 was determined spectrophotometrically (at $\lambda = 542$ nm) from the decrease of its concentration. It was shown that in an acid medium the kinetics of the oxidation of MbO_2 are more sensitive to the presence of Cu(II). Thus a change in the MbO_2:Cu(II) ratio from 1:0.125 to 1:12 increases the rate of reaction by a factor of ~45 at pH 5 and only by a factor of ~15 at pH 7. The steepness of the relations between the logarithm of the rate of reaction and $1/T$ in the temperature range 10–30 °C increases with decrease of pH. The authors explained this by the fact that the interaction of Cu(II) with the His GH1 centre for the binding of Cu(II), which is charged at low pH, leads to local changes unfavourable for electron transfer from haem to this centre, while an increase of temperature eliminates these difficulties. The sharp break in the relation at 30 °C (at temperatures in the range 30–40 °C the relation has much lower slopes, particularly at pH 6 and 7) is explained by a change in the hydration shell of the protein at this temperature. The authors compared the mechanisms of the autoxidation of MbO_2 and the oxidation of MbO_2 by copper(II). In the former case electron transfer via the protein to the O_2 dissolved in the medium as the final acceptor is postulated together with the simple splitting off of O_2^-. In the oxidation by Cu(II) the Cu(II) itself acts as such an acceptor. The electron transfer to copper requires the delocalisation of the electron of haem iron involving the tentative centre for the binding of Cu(II), namely His GH1. The probability of finding an electron in this centre depends on the degree of its protonation. The Cu(II) cation acts analogously—by being bound to His GH1 it increases the positive charge on the histidine, which increases the probability that an electron enters this region. The authors also postulate a mechanism in which Cu(II) is a centre for the binding of the dissolved O_2 acceptor. In this case the Fe(II)...Cu(II)... O_2 electron transfer chain with increasing redox potential may arise, as a result of which the electron transfer becomes thermodynamically spontaneous.

2. The Oxidation of Haemoglobin by Potassium Hexacyanoferrate(III)

The oxidation of haemoglobin and myoglobin by potassium hexacyanoferrate(III) constitutes the usual method of obtaining the oxidised forms of both haemoproteins. Despite this, almost no systematic studies of the kinetics and mechanisms of these reactions have been described in the chemical literature. It is therefore useful to consider only one study, by Antonini et al.[88], in which both of the above human haemoproteins were investigated. It was shown that the simpler system (Mb) reacts with potassium hexacyanoferrate(III) strictly in accordance with a second-order mechanism. $\Delta H^{\neq} = 4$ at pH 6 (0.1 M phosphate buffer) and $\Delta H^{\neq} = 0$ at pH 9.2 (borate buffer). It has been suggested that MbO_2 interacts with hexacyanoferrate(III) only after the dissociation reaction $MbO_2 \rightleftharpoons Mb + O_2$. In this case the rate of oxidation of MbO_2 should be determined either by the rate of its dissociation into Mb and O_2 or by the rate of reaction of Mb with O_2 and hexacyanoferrate(III). A linear relation between the reciprocal of the rate constant and the O_2 concentration in solution was observed at pH 6, which shows that the rate-limiting stage in the process is the dissociation of MbO_2. However, at pH 9.2 the linearity of the above relation no longer holds, which indicates deviations of the process mechanism at pH 9.2 from the simple dissociative scheme. The authors explained the influence of pH on the reaction by its effect not on MbO_2 but on hexacyanoferrate on the grounds that the influence of pH had not been observed in ligand substitution reactions involving MbO_2 (for example, reactions with CO), which have much in common with the reaction involving hexacyanoferrate(III).

When an excess of hexacyanoferrate(III) was allowed to interact with Hb, the first-order rate constant varied linearly during the reaction: it increased by a factor of 1.5 at pH 9.2 and decreased by a factor of 2 at pH 6.45, which was explained by the non-equivalence of the α- and β-chains in Hb. An increase of pH from 6.0 to 9.2 reduced by an order of magnitude the initial rate of reaction. In all cases the rate of oxidation of Hb remained ~100 times lower than the rate of oxidation of Mb under these conditions. The observation that hexacyanoferrate(III) does not oxidise the sulphydryl groups in Hb is significant: the addition of EDTA, which inhibits the oxidation of these groups, did not alter the rate of reaction.

The necessity for the preliminary dissociation into Hb and O_2 is postulated for the reaction of hexacyanoferrate(III) with HbO_2 as well as MbO_2, which was also confirmed by the increase of the rate of oxidation of HbO_2 as the partial pressure of O_2 in the mixture is reduced at pH 6.1 and 9.1 {18 °C, $[HbO_2]_0 = 10^{-5}$ M, and $[K_3Fe(CN)_6]_0 = 5 \times 10^{-3}$ M}.

There exist data concerning the products of the oxidation of horse Hb by hexacyanoferrate(III).[89] It was established by the method of isoelectric focussing that the β-chains are oxidised preferentially. However, a decrease of the ionic strength of the solution (to 0.001 M phosphate) accelerates the oxidation of the α-chains. On the other hand, the addition of inositol hexaphosphate, Cl^-, and the phosphate anion intensifies the oxidation of the β-chains.

3. The Oxidation of Haemoglobin by Nitrite

A much greater number of special investigations have been devoted to the oxidation of haemoglobin by nitrite than to its oxidation by hexacyanoferrate(III). This is apparently because poisoning by nitrite is not rare under modern hygienic conditions. Nitrite contaminates food particularly frequently by being present in water, the sources of which it enters in its turn from the soil[90].

While the mechanism of the reactions of haemoproteins with potassium hexacyanoferrate(III) is relatively simple and at least remains constant during a particular reaction, this cannot be said of the reactions of haemoproteins with nitrite. The most significant characteristic feature of the reaction HbO_2 with nitrite in its autocatalytic character: the kinetic curve is S-shaped (Fig.11). It has been observed[91] that the rate of oxidation of human HbO_2 by nitrite is proportional to $[NaNO_2]^{1.4}$. This reaction order is determined wholly by the structure of the protein, since in the case of rat HbO_2 the order with respect to nitrite is 3–4. Bearing in mind that a decrease of pH from 7.5 to 5.5 entails a linear decrease of the logarithm of the reaction half-life, i.e. that the rate of reaction increases with increase of the activity protons, the authors suggest that the reaction rate depends on the concentration of undissociated HNO_2. At the same time they do not take into account the fact that an increase of the

acidity of the medium always promotes denaturation processes, which have a particularly accelerating effect on the oxidation of HbO_2. Nevertheless, the reality of the increase of the HNO_2 concentration as the pH is reduced to 5.5 must be taken into account and the possible process mechanisms must be considered (HNO_2 can serve, for example, as a source of NO). Next the authors[91] discussed in detail the probability of the involvement of particular system components in the autocatalysis. Special experiments showed that Hb^+ does not influence the rate of reaction and it cannot therefore be the autocatalyst. The same was also established for the possible decomposition products of nitrous acid and also for nitrate. It may be that the autocatalytic character of the reaction is due to the accumulation of hydrogen peroxide in the system, which has been found to shorten the induction period. However, the inertness of H_2O_2 in the subsequent stages of the reaction led the authors to doubt its autocatalytic effect.

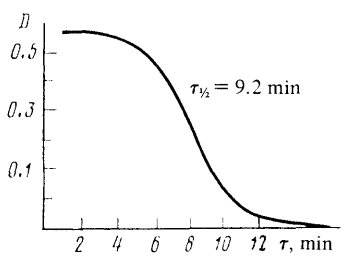


Figure 11. Kinetic curve for the oxidation of HbO_2 by nitrite; $[HbO_2]_0 = 1.8 \times 10^{-5}$ M, $[NaNO_2]_0 = 1.45 \times 10^{-3}$ M; 0.05 M bis–tris buffer at pH 7.0 with 0.1 M NaCl at 25 °C;[100] *D* is the optical density at 578 nm.

The above study[91] was continued by the investigation of the formation of methaemoglobin *in vivo* by administering sublethal amounts of $NaNO_2$ to dogs and rats[90]. After dogs had been administered perorally up to 20 mg of $NaNO_2$ per kilogram of body weight, an increase of the Hb^+ concentration in the blood of the animals was observed in the course of ~30 min up to a concentration of 25%, followed by a slow fall. At the same time EPR showed that in the initial stage of the intoxication process the HbNO concentration rises rapidly and reaches 2% after 10 min and then falls relatively fast. The authors explained the appearance of HbNO by the reduction of the unstable complex Hb^+NO, formed during intoxication, by reductase systems. The possibility of the formation of Hb^+NO enabled the authors to put forward the following mechanism of the oxidation of haemoglobin by nitrite:

$$Fe_p^{2+} + NO_2^- \rightarrow Fe_p^{2+} - NO_2^- ,$$
$$Fe_p^{2+} - NO_2^- + 2H^+ \rightarrow Fe_p^{3+} - NO^+ + H_2O ,$$
$$Fe_p^{2+} - NO^+ \rightleftarrows Fe^{3+} - NO ,$$
$$Fe_p^{2+} - NO + H_2O \rightleftarrows Fe_p^{3+} - H_2O + NO .$$

The second stage explains the observed[91] pH dependence of the oxidation of HbO_2 by nitrite. Stage 3, which is generally speaking fictitious, since it actually indicates only the presence of two resonance structures with Fe(II) and Fe(III), formally demonstrates the mechanism of the oxidation of iron, such oxidation being observed after the substitution of the NO ligand in Fe_p^{3+}–NO by water.

A more elaborate mechanism of the oxidation of HbO_2 by nitrite has been postulated by Tomoda et al.[92]. Bearing in mind that nitrite (like dithionite) is a reductant for O_2, the authors suggested that it interacts with the O_2 bound to iron and generates O_2^-. Experiment confirmed this hypothesis: the introduction of exogenic O_2^- into the system shortened the induction period of the oxidation reaction and the addition of superoxide dismutase lengthened it. The presence of $(\alpha^{O_2}\beta^+)_2$ and $(\alpha^+\beta^{O_2})_2$ was detected by the method involving the isoelectrofocussing of the partly oxidised HbO_2. Taking these two species into account, the process mechanism assumes the following form [Int $Hb(O_2)_2$ represents different half-oxidisied HbO_2 species]:

$$Hb(O_2)_4 + 2NO_2^- \rightarrow Int\,Hb(O_2)_2 + 2NO_3^- + O_2^- ,$$
$$2NO_2^- + 2O_2^- + 6H^+ \rightarrow 2NO + H_2O_2 + 2H_2O + O_2 ,$$
$$Hb(O_2)_4 + 2NO \rightarrow IntHb(O_2)_2 + 2NO_2^- + O_2^- ,$$
$$IntHb(O_2)_2 + 2NO_2^- \rightarrow Hb^+ + 2NO_3^- + O_2^- ,$$
$$2NO_2^- + 2O_2^- + 6H^+ \rightarrow 2NO + H_2O_2 + 2H_2O + O_2 ,$$
$$Int\,Hb(O_2)_2 + 2NO \rightarrow Hb^+ + 2NO_2^- + O_2^- .$$

Thus

$$Hb(O_2)_2 + 2NO_2^- + 6H^+ \rightarrow Hb^+ + 2NO_3^- + H_2O_2 + 2H_2O + O_2 ,$$
$$2O_2^- + H_2O_2 \rightarrow 2H^+ + 2NO_3^- ,$$

or ultimately

$$Hb(O_2)_4 + 4NO_2^- + 4H^+ \rightarrow Hb^+ + 4NO_3^- + 2H_2O + O_2 .$$

The final product of the oxidation of haemoglobin by nitrite has aroused particular interest among investigators. It has been noted[93] that, although the absorption spectra of the Hb^+ solutions formed on oxidation of HbO_2 by nitrite and hexacyanoferrate(III) have the same maximum at 630 nm, in other regions the spectra differ appreciably. The main difference between the nitrite Hb^+ is the presence of a distinct maximum at 540 nm. The hexacyanoferrate(III) Hb^+ has a maximum at 504 nm. The specific spectroscopic characteristics of the nitrite Hb^+ made it necesssary to assume[94] that nitrite oxidises the haem iron and at the same time reacts irreversibly with the protein or with the porphyrin. It was then claimed that the above reactions do not entail significant changes in the Hb^+ structure. The following experiment was carried out to demonstrate the irreversibility of the reaction of HbO_2 with nitrite: Hb^+ which had not been treated with nitrite beforehand was made to react with $NaNO_2$, whereupon NO_2^- was bound to Hb^+ in accordance with a sigmoidal relation; after the dialysis of the product combined with the ligand, it was again treated with nitrite and in this instance the NO_2^- was bound in accordance with a hyperbolic relation. The application of the EPR method[95] demonstrated the paramagnetic nature of the intermediate products of the oxidation of HbO_2 by nitrite. The formation of paramagnetic centres with $g = 2.005$ as well as the oxidation itself were characterised by an induction period and were accelerated with increase of the concentration of nitrite and with decrease of pH. The maximum of the EPR signal corresponded to a break in the Hb^+ formation curve. The EPR signal detected characterises the paramagnetic centres which exhibit autocatalytic activity and are not bound directly to NO. However, in a recently published study[96] it is claimed that prolonged dialysis of the Hb^+ solutions obtained by oxidising HbO_2 and Hb by nitrite and hexacyanoferrate(III) leads to the disappearance of spectroscopic differences betwee Hb^+ of different origin. This enabled the authors to assume that the conformations of Hb^+ are identical in the two cases, which rules out the hypothesis of the oxidation of the protein or the porphyrin fragment in

either procedure for the oxidation of haemoglobin. It is remarkable that the idea of the non-identity of the nitrite and hexacyanoferrate(III) Hb^+, allegedly associated with the different rates of their reduction by ascorbic acid[97], was disproved as early as 1943 [98] after experiments involving 24 h dialysis of the hexacyanoferrate(III) Hb^+ containing, before dialysis, residual potassium hexacyanoferrate(II), which accelerates the reduction of Hb^+.

As noted above[88], whereas hexacyanoferrate(III) oxidises Hb faster than HbO_2, the opposite behaviour obtains in the reaction with nitrite: Hb is not oxidised by nitrite at all[99]. The idea naturally arose that the allosteric effectors which reduce the affinity of Hb for O_2 should inhibit the oxidation of HbO_2 by nitrite. This hypothesis was confirmed in experiments with added inositol hexaphosphate[100]. The latter eliminated almost completely the rapid stage of the process, which follows the induction period. The mechanism of the action of inositol hexaphosphate, which presuposes the conversion of the *R* conformation of haemoglobin into the *T* conformation, has been satisfactorily confirmed by the accelerating influence of this effector on the oxidation of HbO_2 by hexacyanoferrate(III). However, later data[101] showed that other powerful allosteric effectors (DPG, NADP, and ADP) present in 2 and 5 molar excess do not affect the rate of oxidation of Hb by nitrite. It was concluded that the effect of inositol hexaphosphate observed previously is due to some factor other than the $R \rightarrow T$ transition.

The reactive SH groups play a special role in the oxidation of HbO_2 by nitrite[101]. It has been established that the blocking of these groups by iodoacetamide eliminates the inhibiting effect of inositol hexaphosphate for HbO_2 but causes no changes in the case of Hb.

V. THE OXIDATION OF HAEMOGLOBIN UNDER THE INFLUENCE OF RADIATION

In 1927 Fricke and Peterson[102] showed that, in the radiolysis of solutions of HbO_2, the latter undergoes partial oxidation. Manoilov devoted a number of studies to the action of ionising radiation on haemoproteins. He is the author of a review[103] which discusses, in particular, his work and that of other investigators on the oxidation of haemoglobin under the influence of radiation. In view of the existence of this review and also that of Starodub et al.[104], we shall consider only briefly certain studies, which give an idea about this research field and the main results achieved.

In order to understand the causes of the oxidation of haemoglobin, it is important to know the different characteristic changes occurring when solutions are irradiated. In particular a decrease of the redox potential of human haemoglobin has been observed in the course of γ-irradiation[105]. For a dose of 10–100 krad, the changes in the redox potential are slight. The potential falls by 10 mV at 500 krad and by 45 mV at 4.75 krad. The oxidation of HbO_2 on γ-irradiation of its solutions with doses in excess of 1 Mrad is accompanied by a change in the sedimentation coefficient and in the viscosity of the solution[106]. For doses greater than 4–5 Mrad, the appearance of haemochromogen was noted. During irradiation, the affinity of some of the haemoglobin molecules for O_2 increases and the haem–haem interactions are weakened. As stated above[30], these facts characterise the appearance in the solution of partly oxidised forms of haemoglobin.

γ-Irradiation can change from a factor promoting the oxidation of haem iron to one which causes reduction when Hb^+ solutions are irradiated. The solvated electron has been postulated as the primary reducing agent[107,108]:

$$Hb(Fe^{3+})_4 + e^-_{aq} \rightarrow Hb(Fe^{3+})_3(Fe^{2+}).$$

Hb^+ is also reduced on γ-irradiation of its frozen solutions at 77 K.[109] The difference between the optical spectra of the reduced Hb^+ and that of the usual Hb justifies the hypothesis of the formation during the reduction process of an active centre in a low-spin state, the centre being atypical for Hb.

The effects observed in the γ-irradiation of haemoglobin solutions are to a large extent characteristic also of irradiation by X-rays. It has been noted[110] that the stage of the haemoglobin oxidation proper is preceded by a stage in which O_2 is abstracted from HbO_2. The deoxy-form formed resembles the known product of the photodissociation of HbO_2. The Hb^+ arising during irradiation is in a high-spin state. For an irradiation dose of 10–100 krad, a decrease of the redox potential has been noted[111].

Haemoglobin is also oxidised during photoirradiation. During the exposure of an aqueous solution of mouse HbO_2 at 20–50 °C and pH 6.0 to the ultraviolet light of the PRK-4 lamp, the degree of oxidation of haemoglobin increases with the irradiation dose[112]. Under anaerobic conditions there is no oxidation. It has been suggested that the oxidation is preceded by the rupture of the haem–haemoglobin bond.

The damage to haemoglobin during irradiation can therefore be one of the links in the general radiation pathology of the animal organism[103].

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During the preparation of this review for the press, studies on the autoxidation of haemoglobins and myoglobins[113–115], on the oxidation of HbO_2 by phenylhydrazine[116] and nitrofurantoin[117], on the oxidation of haemoglobins by nitrites[118–121], and on the oxidation of sulphhaemoglobin by hexacyanoferrate(III) [122] were published.

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