Oxidation of ascorbic acid in the presence of phthalocyanine metal complexes. Chemical aspects of catalytic therapy of cancer

2.* Catalysis by cobalt octacarboxyphthalocyanine. Reaction products

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The products of ascorbic acid oxidation in the presence of cobalt octa-4,5-carboxyphthalocyanine sodium salt (TPH) were identified. These include the ascorbate radical ($A^{\bullet -}$), hydroxyl radical ($OH^{\bullet}$), and hydrogen peroxide (H_2O_2). The kinetics of accumulation and consumption of the reaction products was studied. For the concentration ranges of ascorbic acid $C_{AH_2} = 0—2.5 \cdot 10^{-3}$ mol L^{-1} and the catalyst $C_{TPH} = 0—3.5 \cdot 10^{-5}$ mol L^{-1}, the the highest possible concentration of the ascorbate radical is $\sim 10^{-7}$ mol L^{-1}, the concentration of H_2O_2 is $7 \cdot 10^{-4}$ (30% of the starting concentration of ascorbic acid) and the concentration of the hydroxyl radical is at most 10^{-6} mol L^{-1}.

Key words: cobalt phthalocyanine, ascorbic acid, oxidation, hydrogen peroxide, ascorbate radical, hydroxyl radical, catalysis, cancer therapy.

Oxidation of ascorbic acid (AH_2) is a highly important process related to vital functions of living organisms. Several years ago this reaction has been used as the basis for developing a new method of cancer therapy.[2] The essence of the method is in that a catalyst for AH_2 oxidation is introduced into the tumor either systemically or regionally, and after a specified period, ascorbic acid is administered systemically into the body. The cytotoxic effect of the catalyst—AH_2 system is assumed to be due to the presence of a free radical formed by the catalytic oxidation of AH_2. However, no systematic study of the nature of these radicals or quantitative characteristics of the processes in which they are formed and consumed has been carried out to date.

Various phthalocyanine metal complexes (PcM) exhibiting enhanced tumor-seeking properties, were studied as catalysts in the catalytic therapy of cancer.[2,3] Among these, the sodium salt of cobalt octa-4,5-carboxyphthalocyanine (Teraphthal (TPH)) proved to be the most efficient in experiments on cells and animals with transplanted tumors.

Previously,[1] we studied the kinetics of oxidation of ascorbic acid in the presence of TPH, obtained the kinetic equation, and proposed a step-by-step reaction mechanism. The aim of this research is to identify the reaction products and to study the kinetics of product accumulation and consumption.

* For Part 1, see Ref. 1.

Experimental

Sodium salt of cobalt octa-4,5-carboxyphthalocyanine $CoPc(COONa)_8$ — Teraphthal (TPH) was synthesized at the Research Institute of Organic Intermediate Products and Dyes and complies with the Enterprise Pharmacopeia Provision (FSP No. 42-0047-1680) approved by the Russian Federation Pharmacopeia Committee. The electronic absorption spectrum of Teraphthal in a 0.025 *M* aqueous phosphate buffer with pH 6.9 has maxima at λ 674 ($\varepsilon = 1.2 \cdot 10^5$ L mol^{-1} cm^{-1}) and 333 nm.

Ascorbic acid (AH_2), pharmaceutical grade (Russia). The electronic absorption spectrum of a solution of the acid in a 0.025 *M* phosphate buffer with pH 6.9 has a maximum at λ = 267 nm, $\varepsilon = 1.4 \cdot 10^4$ L mol^{-1} cm^{-1}.

General procedure for the catalytic oxidation of ascorbic acid has been reported previously.[1] The experimental conditions were as follows: aqueous solutions (phosphate buffer with pH 6.9), room temperature, air. The reaction products are ascorbate radicals, superoxide radical anions, hydroxyl radicals, hydrogen peroxide.

Ascorbate radical. The ascorbate radical ($A^{\bullet -}$) was identified and its concentration was determined by ESR spectroscopy. The spectra were recorded on a Varian E-3 spectrometer

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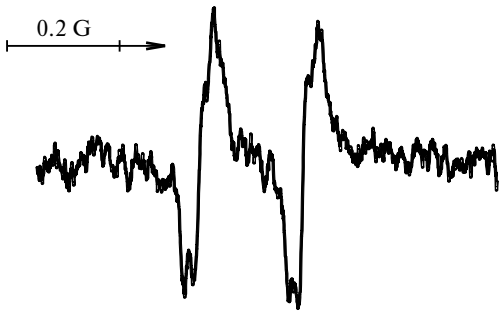

Fig. 1. ESR spectrum of the ascorbate radical $A^{\bullet -}$. Solution in a phosphate buffer, pH 6.9. Conditions of recording the spectra: amplification $5 \cdot 10^5$, power 12.5 mW; modulation 0.2 G; $C_{AH_2} = 2.2 \cdot 10^{-3}$ mol L^{-1}, $C_{TPH} = 0.35 \cdot 10^{-4}$ mol L^{-1}.

(power 6.3—10 mW, amplification $5 \cdot 10^5$, modulation amplitude 1 G, magnetic field sweep 100 G). The resulting spectra (Fig. 1) were similar to those described in the literature; the splitting between the main components in the spectrum (Fig. 1) was 1.8 G, which coincided with published data.[4] The concentration of $A^{\bullet -}$ in the reaction mixture was determined from the ESR signal intensity using the shape factor. The TEMPOL nitroxide was used as the standard for determination of the shape factor.

Superoxide radical anion $O_2^{\bullet -}$. This species was determined by ESR spectroscopy using spin traps. Tiron (sodium 4,5-dihydroxy-1,3-benzodisulfonate) was employed as the trap. The spectra were recorded on an X-range Varian EPR E-4 spectrometer (power 10 mW, amplification $5 \cdot 10^3$, modulation amplitudes 1 and 0.25 G, magnetic field sweep 100 G).

Hydroxyl radical. The $OH^{\bullet -}$ radical was detected using a method based on oxidation of 2-desoxyribose (DR).[5] Preliminarily, it was shown that the addition of DR to the reaction mixture does not influence the oxidation kinetics of AH_2 in the presence of TPH. This method permits determination of the total amount of the $OH^{\bullet}$ radicals formed by the time instant t.

Hydrogen peroxide. 10-Acetyl-3,7-dihydroxyphenoxazine reacts with hydrogen peroxide in 1 : 1 ratio in the presence of peroxidase to give resorufin, which can be easily detected based on the electronic absorption spectrum ($\lambda_{max} = 563$ nm).[6] It was shown by special experiments that this reaction is not affected by the presence of ascorbic acid and that the product (resorufin) does not undergo any transformations under these conditions.

Results and Discussion

According to the mechanism proposed previously[1] for the oxidation of ascorbic acid in the presence of TPH, this may yield the ascorbate radical ($HA^{\bullet}$), which exists in the dissociated form (pK 4.25),[7] *i.e.*, as the $A^{\bullet -}$ radical anion, in the pH range under study, the superoxide radical anion $O_2^{\bullet -}$, the hydroxyl radical $OH^{\bullet}$, hydrogen peroxide, and dehydroascorbic acid (A). The last-mentioned product was not studied in our work.

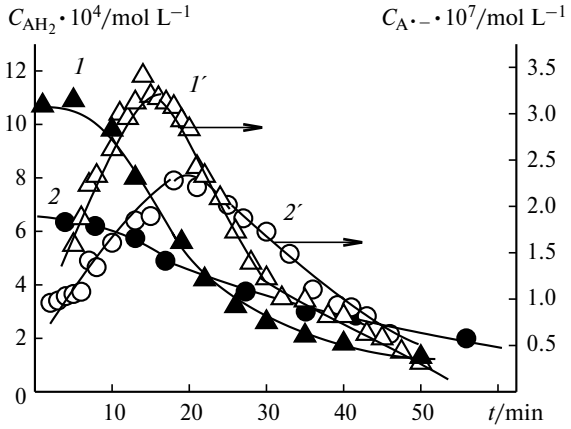

Fig. 2. Typical kinetic curves for the oxidation of ascorbic acid (*1*, *2*) and the formation of the ascorbate radicals (*1′*, *2′*) in the AH_2—TPH system; $C_{TPH} = 0.35 \cdot 10^{-4}$ mol L^{-1}, C_{AH_2}, mol L^{-1}: $1.06 \cdot 10^{-3}$ (*1*); $6.5 \cdot 10^{-4}$ (*2*); pH 6.9, air, room temperature.

Ascorbate radical $A^{\bullet -}$

The concentrations of the radical anion $A^{\bullet -}$ were measured in two series of experiments in which the initial concentration of either the substrate or the catalyst was varied within the ranges indicated above, while the concentration of the second component remained constant. Figure 2 presents kinetic curves for the variation of the concentration of $A^{\bullet -}$ during the experiment and the consumption curves of the ascorbic acid. The pattern of the variation of $A^{\bullet -}$ concentration indicates that this is a reaction intermediate. Nevertheless, the fact that the amount of the $A^{\bullet -}$ radical anion formed is three orders of magnitude lower than the amount of the consumed acid is less obvious. Analysis of the published data and the kinetic results obtained previously[1] suggests that the major process giving rise to this radical anion over the given range of parameters is the oxidation of the ascorbic acid monoanion (AH^-) with dioxygen coordinated to the cobalt atom of the Teraphthal

$$HA^- + TPH \cdot O_2 \rightarrow A^{\bullet -} + O_2^{\bullet -} + H^+ + TPH, \qquad (1)$$

while the consumption of the radical anion follows the route

$$A^{\bullet -} + A^{\bullet -} + 2\,H_2O \rightarrow AH_2 + A + 2\,OH^-, \qquad (2a)$$

$$O_2 + A^{\bullet -} \rightarrow A + O_2^{\bullet -}. \qquad (2b)$$

However, the rate constant for reaction (2b) is four orders of magnitude lower than that for pathway (2a);[1] hence, the consumption of the ascorbate radical for the reaction with oxygen can be excluded from consideration. In this case, the kinetic equations for the accumulation

and consumption of $A^{\bullet -}$ at a fixed partial pressure of dioxygen have the following form:

$$W_{form} = k_{form} C_{AH_2} C_{TPH}, \quad (3)$$

$$W_{cons} = k_{cons} C^2_{A^{\bullet -}}, \quad (4)$$

where k_{form}, k_{cons} are the rate constants for the formation and consumption of the $A^{\bullet -}$ radical anion, respectively.

Within the framework of this assumptions, the rate of variation of the concentration of the radical anion is equal to the algebraic sum of the formation and consumption rates

$$W_{obs} = W_{form} - W_{cons} = k_{form} C_{AH_2} C_{TPH} - k_{cons} C^2_{A^{\bullet -}}, \quad (5)$$

and at the point of maximum, $W_{form} = W_{cons}$

Processing of the ascending and descending branches of the kinetic curves and the points corresponding to the position of the maximum given by Eq. (5) showed that the curves for variation of the concentration of the ascorbate radical during the oxidation of ascorbic acid are described satisfactorily by Eq. (5). This made it possible to calculate the rate constants for reactions (3) and (4): k_{form} = 0.01 L mol^{-1} s^{-1}, $k_{cons} \approx 5 \cdot 10^3$ L mol^{-1} s^{-1}. The only rate constant for the recombination of ascorbate radicals (reaction (2)) reported in the literature,[7] equal to $7 \cdot 10^4$ L mol^{-1} s^{-1}, differs from the value we found by almost an order of magnitude. In our opinion, this difference is not essential and may be related to either different experimental conditions (pH, temperature mode, and so on) or to other reactions consuming the ascorbate radical. The k_{form} value proved to be three orders of magnitude lower than the rate constant for the oxidation of ascorbic acid (k_{AH_2} = 30 L mol^{-1} s^{-1}) calculated from kinetic data[1] at an atmospheric pressure of oxygen. This fact may indicate that the major pathway of the catalytic reaction we study is the oxidation of ascorbic acid to give dehydroascorbic acid in the coordination sphere of the TPH metal atom without escape of the intermediate radical anion $A^{\bullet -}$ into the solution bulk.

Superoxide radical anion $O_2^{\bullet -}$

The addition of a spin trap (Tiron) into a solution containing ascorbic acid and a catalyst does not give rise to a signal for the product of reaction between $O_2^{\bullet -}$ and the trap over the whole range of reactant concentrations. This fact is consistent with the data[8] according to which $O_2^{\bullet -}$ can be detected in "substituted Co phthalocyanine (including TPH)—AH_2" systems only after the introduction of KCN whose anion replaces the superoxide from the coordination sphere of cobalt.

This result also does not contradict the above hypothesis according to which the main bulk of ascorbic acid is converted within the coordination sphere of the metal atom without escape of free radicals, possible primary intermediates, into the solution bulk.

The reaction of $A^{\bullet -}$ with $O_2^{\bullet -}$ (reaction (6)) is known[9] to proceed at a high rate even in solution. Therefore, it can be reasonably assumed that the rate of the reaction between these radicals should be even higher in the metal coordination sphere.

$$A^{\bullet -} + O_2^{\bullet -} + H_2O \rightarrow A + HO_2^- + OH^-, \quad (6)$$

$k = 2.6 \cdot 10^8$ L mol^{-1} s^{-1}.

Hydrogen peroxide and the $HO^{\bullet}$ radical

Hydrogen peroxide. The question of formation of hydrogen peroxide upon the oxidation of ascorbic acid in the presence of TPH is a key issue for the understanding of the reaction mechanism and the therapeutic action of this complex. Our experiments have shown that during the process, hydrogen peroxide is accumulated in the reaction zone (subsequently it is slowly consumed) in concentrations comparable, in the order of magnitude, with the concentrations of ascorbic acid (Fig. 3).

$$AH_2 + O_2 \rightarrow A + H_2O_2 \quad (7)$$

The position of the maximum in the kinetic curves for H_2O_2 formation coincides, most often, with the time of complete consumption of ascorbic acid. At low concentrations of TPH (at a constant C_{AH_2} concentration, this corresponds to a high substrate : catalyst ratio), the maximum shifts toward residual concentrations of the acid substantially differing from zero. This is due to the fact that oxidation of ascorbic acid is accompanied by the

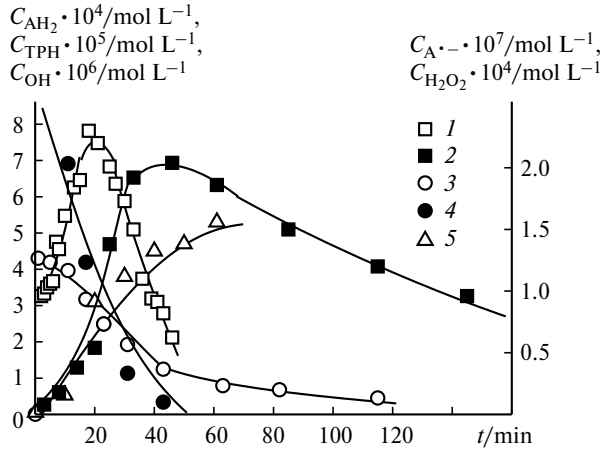

Fig. 3. Kinetic curves for the accumulation and consumption of H_2O_2, oxidation of ascorbic acid, destruction of TPH, generation of the $A^{\bullet -}$ radical anion and the $OH^{\bullet}$ radical (integral curve); $C_{AH_2} = 8.2 \cdot 10^{-4}$ mol L^{-1}, $C_{TPH} = 0.35 \cdot 10^{-4}$ mol L^{-1}, pH 6.9, air.

destruction of the catalyst. Full destruction at low concentrations results in termination of the catalytic process long before the substrate is exhausted.

The concentration of H_2O_2 at the point of maximum increases with an increase in the initial concentration of AH_2; however, it is always substantially lower than the concentration of the converted acid, *i.e.*, processes resulting in hydrogen peroxide consumption occur in parallel with the formation of hydrogen peroxide in the system.

One process consuming H_2O_2 is its catalytic decomposition in the presence of TPH. Special experiments have shown that TPH has a rather high catalytic activity in this reaction. For $C_{H_2O_2} = (1.2—20) \cdot 10^{-4}$ mol L^{-1} and $C_{TPH} = (0.1—1.6) \cdot 10^{-4}$ mol L^{-1}, the initial reaction rate is described by the kinetic equation

$$W_{H_2O_2} = k_{H_2O_2} C_{H_2O_2} C_{TPH}, \quad (8)$$

where $k_{H_2O_2} = 2.3$ L mol^{-1} s^{-1}.

For the reaction catalyzed by the Fe^{3+} ion, $k = 8.8 \cdot 10^{-2}$ L mol^{-1} s^{-1}, while for catalase, $k = 3.0 \cdot 10^7$ (pH 4.5—9).[10]

During decomposition of hydrogen peroxide, the catalyst also undergoes destruction. The kinetic equation in the same concentration range has the form

$$W_{TPH} = k_{TPH} C_{H_2O_2} C_{TPH}, \quad (9)$$

where $k_{TPH} = 0.7$ L mol^{-1} s^{-1}.

The ratio $k_{H_2O_2} : k_{TPH} = 3.3$. This means that one TPH molecule has time to perform, on average, only three catalytic cycles before it decomposes.

The consumption of hydrogen peroxide still continues when the catalyst concentration becomes negligibly small (see Fig. 3). We suggested and confirmed experimentally for the H_2O_2—A system that this effect is due to the reaction of H_2O_2 with dehydroascorbic acid. In the concentration ranges $C_{H_2O_2} = (0.1—3.0) \cdot 10^{-3}$ mol L^{-1} and $C_A = (0.2—2) \cdot 10^{-3}$ mol L^{-1}, the reaction rate is described by the equation

$$W_{H_2O_2} = k_{H_2O_2} C_{H_2O_2} C_A, \quad (10)$$

where $k_{H_2O_2} = 0.22$ L mol^{-1} s^{-1}.

In the system containing all three components (A, H_2O_2, and TPH), the rate of catalyst destruction does not change and the rate of consumption of hydrogen peroxide is the sum of the rates of its catalytic decomposition and its reaction with A (Table 1). Thus, the products of TPH destruction do not exhibit catalytic activity in the decomposition of hydrogen peroxide. The products formed in the reaction of dehydroascorbic acid with hydrogen peroxide were beyond the scope of our study. According to published data,[11] this acid is unstable in aqueous solutions; about 40 products were found by HPLC. To our knowledge, no other information has been reported.

Table 1. Rates of hydrogen peroxide decomposition ($W_{H_2O_2}$) and catalyst destruction (W_{TPH}) in reaction mixtures of various compositions

Composition of the mixture	$W_{H_2O_2} \cdot 10^7$	$W_{TPH} \cdot 10^8$
	mol L^{-1} s^{-1}	
TPH—H_2O_2	1.3	2.0
A—H_2O_2	2.7	—
TPH—A—H_2O_2	3.3	2.7

Note. $C_{TPH} = 0.35 \cdot 10^{-4}$ mol L^{-1}, $C_A = 1.2 \cdot 10^{-3}$ mol L^{-1}, $C_{H_2O_2} = 1.0 \cdot 10^{-3}$ mol L^{-1}.

Hydroxyl radical. The formation of the HO$^\bullet$ radical upon the oxidation of ascorbic acids in the presence of TPH was detected over the whole range of reactant concentrations by the method described in the Experimental, which is based on the use of 2-deoxyribose (see Fig. 3). It can be assumed with sufficient grounds that the main bulk of the radicals is formed upon decomposition of H_2O_2 in the presence of TPH. Therefore, its maximum possible concentration by the end of the major reaction, taking into account the residual content of H_2O_2 in solution, its consumption in the reaction with A, and catalytic decomposition, should be equal to $\sim 3 \cdot 10^{-4}$ mol L^{-1} (for $C^{init}_{AH_2} = 1 \cdot 10^{-3}$ mol L^{-1}). However, the $C_{\Sigma HO\cdot}$ value determined in our experiments did not exceed 10^{-5} mol L^{-1} (see Fig. 3). This may be due to two reasons, namely, the presence of a nonradical pathway for hydrogen peroxide decomposition and competing consumption of HO$^\bullet$ along pathways characterized by higher rate constants than the reaction with 2-desoxyribose (recombination, reaction with ascorbic acid, and so on). The rate constants for these and other reactions involving the hydroxyl radical are of the order of 10^9—10^{10} L mol^{-1} s^{-1}.

The results we obtained provide the conclusion that the oxidation of ascorbic acid with dioxygen in the presence of TPH gives H_2O_2 in a nearly equivalent amount with respect to the consumed ascorbic acid. The intermediate radical products ($O_2^{\bullet -}$ and the major part of $A^{\bullet -}$) are subsequently converted within the coordination sphere of the cobalt atom without leaving to the reaction bulk. The resulting hydrogen peroxide is partially consumed in the reactions with the AH_2 oxidation product (dehydroascorbic acid) and in the catalytic decomposition. The latter process is accompanied by the formation of HO$^\bullet$ radicals; however, the amount of HO$^\bullet$ radicals detectable in our experiments was much lower than the theoretically possible value.

When considering the results from the standpoint of chemical grounds of the catalytic therapy of cancer, one can conclude that the hydroxyl radical and hydrogen

peroxide are among the key cytotoxic agents in the Teraphthal—ascorbic acid system. The concentration of hydrogen peroxide is comparable with that of ascorbic acid, and its ability to destroy biological structures is well-known.

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