ISSN 1068-1620, Russian Journal of Bioorganic Chemistry, 2021, Vol. 47, No. 1, pp. 299–306. © Pleiades Publishing, Ltd., 2021.
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Gallocyanine as a Fluorogen for the Identification of NADPH-Dependent Production of Superoxide Anion Radical by Blood Cells

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Received April 9, 2020; revised April 18, 2020; accepted April 21, 2020

Abstract—The interaction of the oxazine dye gallocyanine with reactive oxygen ($^{\bullet}O_2^-$, H_2O_2) and halogen (HOCl) species has been studied by spectrophotometry, mass spectrometry, and spectrofluorimetry. It has been shown that gallocyanine reacts with HOCl and $^{\bullet}O_2^-$ (but not with H_2O_2) to form fluorescent products. It has been found using the inhibition assay that, both in the xanthine/xanthine oxidase system and in activated human blood neutrophils, the main contribution to the conversion of gallocyanine to a fluorophore comes from its reaction with $^{\bullet}O_2^-$. The results obtained suggest that gallocyanine can act as a fluorogenic chemosensor and be used for estimating the activation of neutrophils and the NADPH-dependent production of superoxide anion radical by neutrophils and other blood cells, as well as for testing antioxidant drugs designed with the aim to correct diseases associated with oxidative stress.

Keywords: gallocyanine, reactive oxygen species, reactive halogen species, oxidative stress, superoxide anion radical, hypochlorous acid, fluorescence

DOI: 10.1134/S1068162021010179

INTRODUCTION

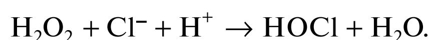
Neutrophils are the most abundant subspecies of human blood granulocytic leukocytes (up to ~70%), which plays a key role in the implementation of the cellular link of innate immunity mainly owing to the production of reactive oxygen species (ROS) and reactive halogen species (RHS). These compounds not only act as mediators of important intra- and intercellular redox-sensitive signaling pathways such as ionic permeability, apoptosis, netosis, and receptor interactions but also, being highly reactive species, kill pathogens (bacteria, viruses, fungi, etc.), performing the antimicrobial function [1]. Enzymes that are mainly responsible for the production of ROS and RHS in neutrophils are NADPH oxidase (EC 1.6.3.1), superoxide dismutase (SOD, EC 1.15.1.1), and myeloperoxidase (MPO, EC 1.11.2.2). The activation of neutrophils in response to a proinflammatory stimulus is accompanied by the assembly of a NADPH-oxidase complex, which catalyzes the reduction of molecular oxygen (O_2) to superoxide anion radical ($^{\bullet}O_2^-$) [2]:

$$NADPH + 2O_2 \rightarrow NADP^+ + 2^{\bullet}O_2^- + H^+. \quad (1)$$

As a result of $^{\bullet}O_2^-$ dismutation (spontaneous or catalyzed by SOD), hydrogen peroxide (H_2O_2) forms:

$$2^{\bullet}O_2^- + 2H^+ \rightarrow O_2 + H_2O_2, \quad (2)$$

which is a substrate of MPO, an enzyme of azurophilic granules of neutrophils [1, 3]. In the human body, MPO catalyzes mainly the oxidation of chloride to hypochlorous acid (HOCl):



$$H_2O_2 + Cl^- + H^+ \rightarrow HOCl + H_2O.$$

ROS and especially RHS (including HOCl), which form during the activation of neutrophils and possess high reactivity, react with many biologically important molecules (nucleic acids, proteins, lipids, antioxidants, and others) and destroy cells and tissues of the

Abbreviations: 4-ABAH, 4-aminobenzoic acid hydrazide; CP, ceruloplasmin; fMLP, *N*-formyl-methionyl-leucyl-phenylalanine; MPO, myeloperoxidase; PMA, phorbol 12-myristate 13-acetate; SOD, superoxide dismutase; RHS, reactive halogen species; ROS, reactive oxygen species; PBS, phosphate-buffered saline.

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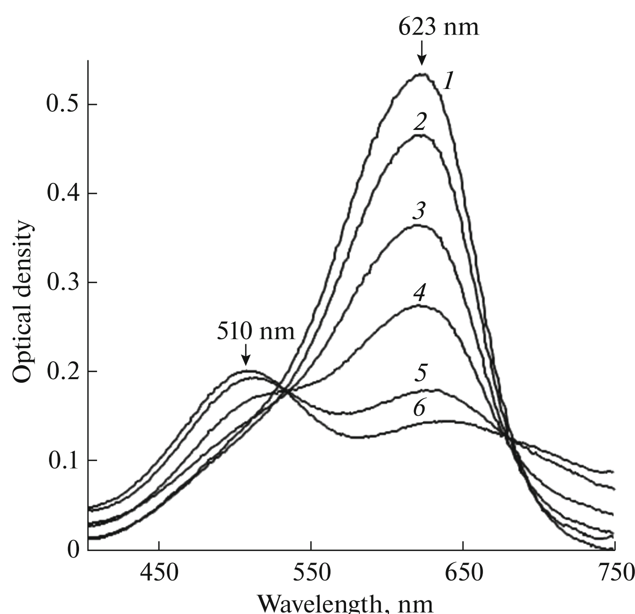

Fig. 1. Absorption spectra of gallocyanine before and after the addition of KO_2. Measuring medium PBS-2, pH 7.4 (see Experimental). Gallocyanine concentration 20 μM. Concentration of KO_2 (mM): (*1*) 0; (*2*) 0.22; (*3*) 0.76; (*4*) 0.98; (*5*) 1.31; (*6*) 1.64.

body, inducing oxidative/halogenative stress, which provokes the development of inflammatory diseases: cardiovascular, neurodegenerative, oncological, and others [3–5]. It becomes clear how important it is to control and estimate the activity of enzymes responsible for the production of ROS and RHS. It is obvious that the registration of ROS/RHS production by cells enables the development of new methods and approaches aimed at the prophylaxis, diagnosis, and monitoring of the efficacy of the therapy of diseases associated with oxidative/halogenative stress. Among the methods used for the detection of ROS and RHS (mass spectrometry in combination with different types of chromatography, enzyme-linked immunosorbent assay, chemiluminescence, spin traps, and others), fluorescence analysis is distinguished for high sensitivity and availability [6–8].

A great number of low-molecular-weight fluorogenic probes have been synthesized recently, which make possible, among other things, the real-time recording of the production of various ROS and RHS both inside and outside the cell: 2',7'-dichlorodihydrofluorescein diacetate, dihydrorhodamine 123, 10-acetyl-3,7-dihydroxy phenoxazine (Amplex Red), aminophenyl fluorescein (2-[6-(4'-amino)phenoxy-3*H*-xanthen-3-on-9-yl]benzoic acid), and others [6–11]. However, these probes are not always sufficiently sensitive and specific in the analysis of some ROS/RHS; their synthesis is often very laborious, which substantially increases their price, or they are simply inconvenient to use in the analysis (poor solubility in water, insufficient stability, etc.). Thus, 2',7'-dichlorodihydrofluorescein diacetate, dihydrorhodamine 123, and aminophenyl fluorescein are equally well oxidized by free radicals, peroxynitrite, and HOCl [6, 9]. Amplex Red, which is designed to identify H_2O_2 in the presence of peroxidases, is easily oxidized by RHS. Moreover, reducing agents such as NADH and glutathione can significantly distort the results of analysis [10, 11].

We have developed previously a kinetic spectrophotometric method for measuring the halogenating activity of MPO, which is based on the bleaching of the oxazine dye celestine blue B by the action of RHS [12]. Later, we have shown that this dye can be successfully applied to identify HOCl produced by activated neutrophils using flow cytometry and confocal microscopy [13].

In the present study, we focused our attention on another oxazine dye, gallocyanine (4-hydroxy-7-dimethylamino-1-carboxyphenoxazin-3 chloride). It is a structural analog of celestine blue B but features a higher photostability and relatively good solubility in PBS and is easily synthesized with a yield close to quantitative. Owing, among other things, to these properties, gallocyanine has been used for more than half a century in the histological analysis for staining nuclei [14] and quantitative determination of nucleic acids [15]. We studied the interaction of gallocyanine with the main ROS ($^{\bullet}O_2^-$, H_2O_2) and RHS (HOCl) with the aim to use the dye for the identification of the production of these compounds by activated neutrophils.

RESULTS AND DISCUSSION

Interaction of Gallocyanine with $^{\bullet}O_2^-$. It is known that potassium dioxide (KO_2) in an aqueous solution is hydrolyzed to form a short-lived superoxide anion radical:

$$KO_2 + H_2O \rightarrow H^+ + KOH + {}^{\bullet}O_2^-, \quad (3)$$

which is rapidly dismutated to hydrogen peroxide according to reaction (2). The overall equation for the reaction looks like this:

$$2KO_2 + 2H_2O \rightarrow 2KOH + H_2O_2 + O_2. \quad (4)$$

To study the interaction of gallocyanine with $^{\bullet}O_2^-$, a weighed sample of dry KO_2 was added to a gallocyanine solution, the mixture was stirred, and absorption spectra in the visible region were recorded. The results of measurements are shown in Fig. 1. It is seen that the absorption maximum of the gallocyanine solution is at 623 nm. As the concentration of added KO_2 is increased, the height of this peak decreases. Simultaneously, an absorption peak with a maximum at 510 nm appears, its intensity increases, and the color of the solution changes from blue to pale violet. Taken together, this indicates the utilization of gallocyanine

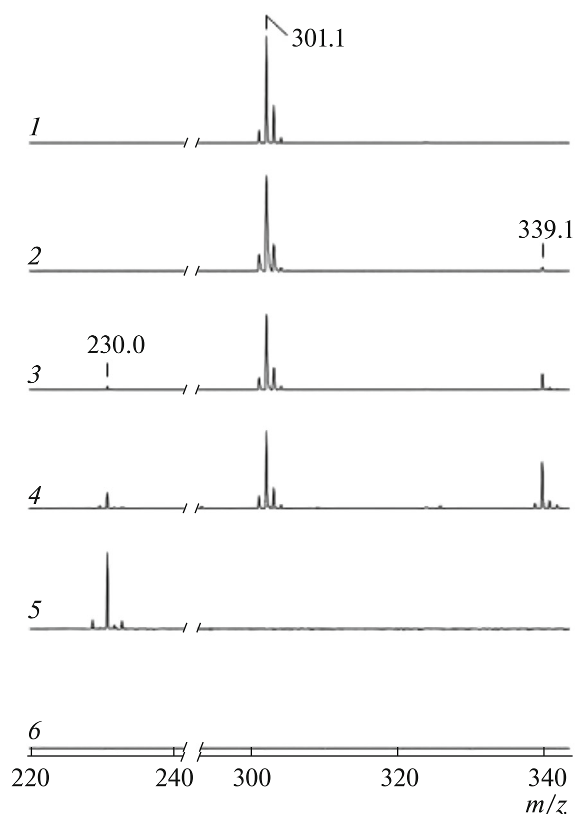

Fig. 2. Mass spectra of gallocyanine before and after the addition of KO_2. Measuring medium PBS-2, pH 7.4. Gallocyanine concentration 80 μM. Concentration of KO_2 (mM): (*1*) 0; (*2*) 1.14; (*3*) 2.84; (*4*) 5.69; (*5*) 11.38; (*6*) mass spectrum of the matrix (2,5-dihydroxybenzoic acid) in the absence of gallocyanine.

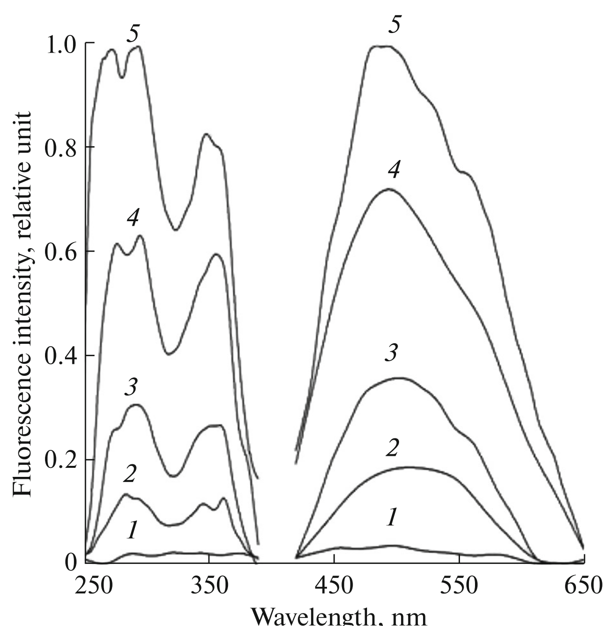

Fig. 3. Excitation and emission spectra of gallocyanine fluorescence before and after the addition of KO_2. Measuring medium PBS-1, pH 7.4. Gallocyanine concentration 5 μM. Concentration of KO_2 (mM): (*1*) 0; (*2*) 0.7; (*3*) 1.6; (*4*) 2.7; (*5*) 4.1. Excitation at 360 nm, emission at 490 nm.

and the formation of at least one product. We showed in control experiments that the addition of a H_2O_2 solution (10–1000 μM), which forms according to reaction (4), to gallocyanine (20 μM) does not lead to changes in its absorption spectrum (data not shown). This indicates that gallocyanine does not react with H_2O_2. Thus, it may be concluded that the change in the absorption spectrum of gallocyanine after the addition of KO_2 is caused by its reaction with $^{\bullet}O_2^-$, which forms as an intermediate during the hydrolysis of KO_2 according to reaction (3).

This fact is supported by the mass spectra of gallocyanine recorded before and after the addition of KO_2 at different concentrations (Fig. 2). The mass spectrum of gallocyanine (spectrum *1* in Fig. 2) is a monoisotopic distribution; the most intense peak (*m/z* 301.1) corresponds to the mass of the cation $[C_{15}H_{13}N_2O_5]^+$, which consists of the main isotopes (^{12}C, ^{1}H, ^{14}N, ^{16}O). As the KO_2 concentration increases, a signal at *m/z* 339.1 appears (spectrum *2* in Fig. 2), increases (spectra *3* and *4*), and then disappears (spectrum *5*). In parallel, beginning at a KO_2 concentration of 2.84 mM, a signal at *m/z* 230.0 appears and increases. At a KO_2 concentration of 11.38 mM (spectrum *5* in Fig. 2), the peak from the initial gallocyanine (*m/z* 301.1) completely disappears, and only the signal at *m/z* 230.0 remains. This result can be explained on the assumption that, in the reaction of gallocyanine with KO_2, first a product at *m/z* 339.1 forms, which finally is converted to a compound with *m/z* 230.0. At least one of the products is a fluorophore, which is confirmed by the results given in Fig. 3 from which it is seen that the addition of KO_2 to a gallocyanine solution leads to the appearance of fluorescence, which increases with increasing KO_2 concentration (maxima in the region of 260–370 nm in the excitation spectrum and in the region of 480–500 nm in the emission spectrum; Fig. 3).

In addition to KO_2, we used xanthine oxidase for $^{\bullet}O_2^-$ production; in the presence of xanthine, this enzyme catalyzes the reduction of O_2 to $^{\bullet}O_2^-$ [16]. The results of the experiment are shown in Fig. 4. It is seen that, in the presence of xanthine oxidase and its substrate xanthine, an increase in fluorescence intensity at 490 nm occurs (excitation at 360 nm) (curve *1* in Fig. 4), whereas in the absence of xanthine, the enzymatic reaction does not proceed, and there is no increase in fluorescence (curve *2* in Fig. 4). This result indicates that $^{\bullet}O_2^-$ synthesized in the enzymatic reaction (see reaction (1)) interacts with gallocyanine to form a fluorophore. It is evident from the data in Fig. 5 that the

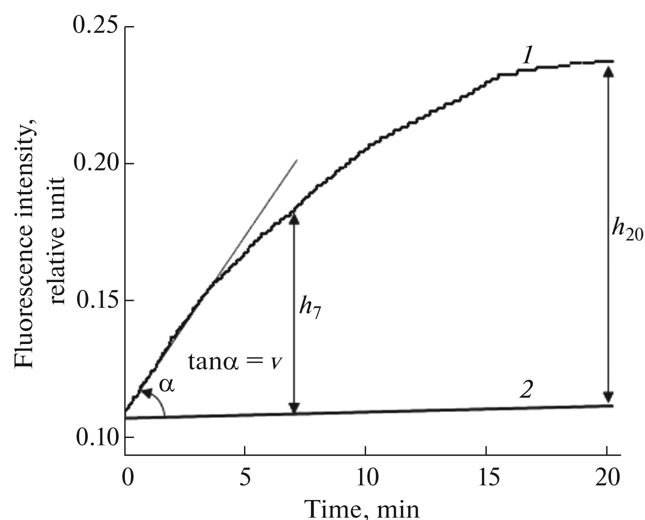

Fig. 4. Kinetics of changes in gallocyanine fluorescence intensity in the xanthine/xanthine oxidase system (curve *1*). The concentration of gallocyanine 5 μM, of xanthine 1 mM, and of xanthine oxidase 0.52 μM. Measuring medium PBS-1, pH 7.4. Curve *2,* a control curve obtained in the absence of xanthine. Excitation at 360 nm, emission at 490 nm. The figure shows the parameters under study: the fluorescence intensity compared with the background level 7 (h_7) and 20 (h_{20}) min after the initiation of the reaction and the rate of the chemical transformation of gallocyanine (v).

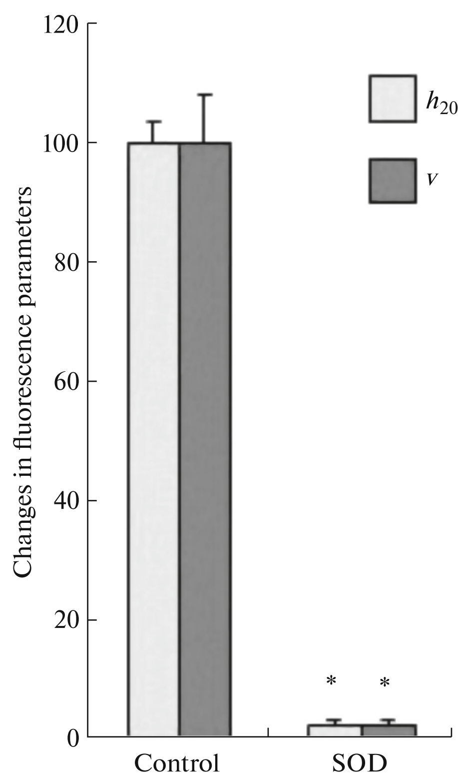

Fig. 5. Effect of SOD (1.6 μM) on the intensity (h_{20}) and the rate of fluorescence buildup (v) in the gallocyanine + xanthine/xanthine oxidase system. The concentration of gallocyanine 5 μM, of xanthine 1 mM, and of xanthine oxidase 0.52 μM. Measuring medium PBS-1, pH 7.4. Excitation at 360 nm, emission at 490 nm.

fluorescence intensity (h_{20}) and the rate of the chemical transformation of gallocyanine (v) in the xanthine/xanthine oxidase system are almost entirely inhibited in the presence of SOD. This fact indicates that $^{\bullet}O_2^-$ makes a decisive contribution to the chemical conversions of the dye.

Interaction of gallocyanine with HOCl. Figure 6 shows the absorption spectra of gallocyanine before and after the addition of HOCl at different concentrations to a gallocyanine solution. It is seen that the gallocyanine solution has an absorption maximum at 623 nm (spectrum *1* in Fig. 6a). The molar extinction coefficient calculated from this spectrum is ε_{623} = 28000 M^{-1} cm^{-1}, which agrees well with the literature data [17, 18]. The addition of HOCl to the dye led to a decrease in the optical density of the solution at 623 nm and the appearance of a less intense absorption band with a maximum at 475 nm (Fig. 6a), indicating the formation of a reaction product. It is seen from the inset in Fig. 6a that the decrease in the gallocyanine concentration is proportional to the concentration of added HOCl so that the coefficient of HOCl : gallocyanine stoichiometry is ~1.5. However, as seen from Fig. 6b, further increase in HOCl concentration leads to a decrease in the optical density of the solution at 475 nm and the appearance of an absorption band at 416 nm. During the reaction, the blue-violet color of the initial gallocyanine solution first changes to orange, and upon further increase in HOCl concentration the solution is decolorized. This result indicates that HOCl reacts with gallocyanine to form at least two products of which the first, which has an absorption band at 475 nm, is an intermediate and at higher HOCl concentrations is converted to a product that absorbs at 416 nm.

This conclusion is confirmed by the mass spectra of gallocyanine (Fig. 7) recorded before and after the addition of HOCl at different concentrations to the gallocyanine solution. It is seen from Fig. 7 that, as the HOCl concentration increases, the intensity of the signal corresponding to the mass of the cation of initial gallocyanine $[C_{15}H_{13}N_2O_5]^+$ (*m*/*z* 301.1) gradually decreases. Concurrently, signals at *m*/*z* 328.3 and 452.0 appear, which, most likely, correspond to two products of the reaction of HOCl with gallocyanine. At least one of these products is a fluorophore. This is evidenced by the fluorescence excitation and emission spectra of the gallocyanine solution after the addition of HOCl at different concentrations (Fig. 8). It is seen from Fig. 8 that, with increasing concentration of HOCl added to gallocyanine, the fluorescence intensity of the dye solution increases (maxima at 269, 292, and 360 nm in the excitation spectrum and at 490 nm

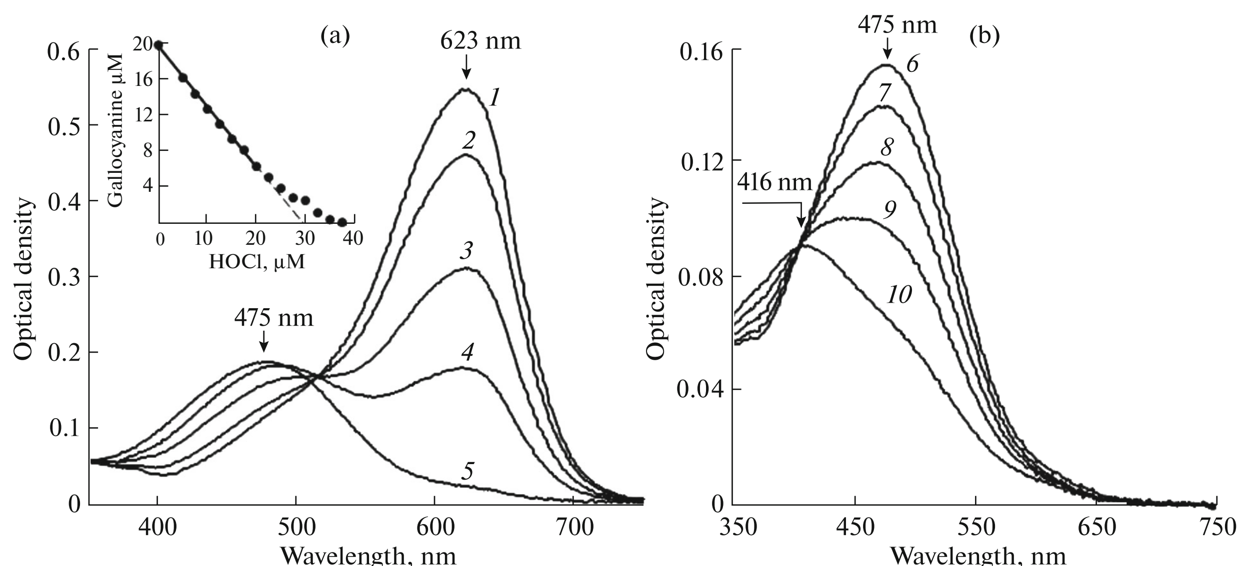

Fig. 6. Absorption spectra of gallocyanine before and after the addition of HOCl. Measuring medium PBS-2, pH 7.4. Gallocyanine concentration 20 μM. The concentration of HOCl (μM) (a): (*1*) 0; (*2*) 7.5; (*3*) 15.0; (*4*) 22.5; (*5*) 40.0; (b): (*6*) 45.0; (*7*) 52.5; (*8*) 60.0; (*9*) 67.5; (*10*) 75.0. On the inset is the dependence of the drop in the concentration of gallocyanine in solution on the concentration of added HOCl, calculated from the decrease in the optical density at 623 nm.

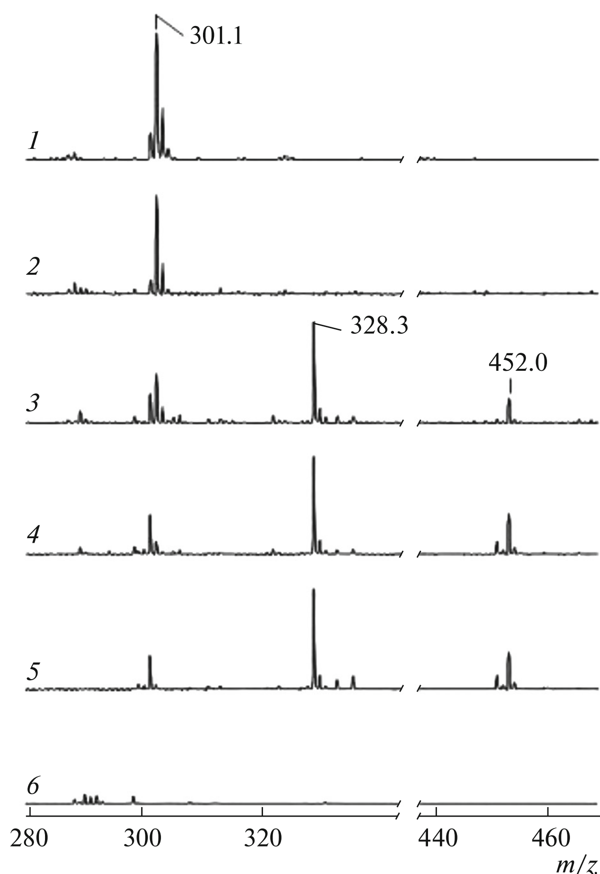

Fig. 7. Mass spectra of gallocyanine before and after the addition of HOCl. Measuring medium PBS-2, pH 7.4. Gallocyanine concentration 80 μM. The concentration of HOCl (mM): (*1*) 0; (*2*) 0.4; (*3*) 4.0; (*4*) 8.0; (*5*) 16.0; (*6*) a mass spectrum of the matrix (2,5-dihydroxybenzoic acid) in the absence of gallocyanine.

in the emission spectrum, Fig. 8), which is due to the formation of the reaction product.

Thus, both reagents examined ($^{\bullet}O_2^-$ and HOCl) react with gallocyanine in a cell-free medium to form fluorescent products. Then, we tried to elucidate whether gallocyanine can be used as a fluorogenic chemosensor for the registration of ROS and/or RHS production by human blood neutrophils.

Production of $^{\bullet}O_2^-$ by neutrophils. It is known that, among all blood cells, neutrophils are the main carriers of MPO and NADPH oxidase. The stimulation of neutrophils leads, on the one hand, to the assembly and activation of the NADPH oxidase enzyme complex, which is accompanied by enhanced production of $^{\bullet}O_2^-$ [2], and, on the other hand, to cell degranulation, secretion of MPO from azurophilic granules into the extracellular space, and, as a consequence, the synthesis of HOCl [1, 4]. As demonstrated by the above results, both events should contribute to the transformation of gallocyanine to a fluorogenic form. To determine which of the enzymatic systems of neutrophils is responsible to a greater extent for the transformation of gallocyanine to a fluorophore, we activated neutrophils by adding *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) or phorbol 12-myristate 13-acetate (PMA) in the presence of gallocyanine and various substances capable of both scavenging and/or eliminating ROS/RHS and inhibiting their production, while recording changes in fluorescence intensity. The results of experiments are given in Table 1.

It is evident from the data in Table 1 that 4-aminobenzoic acid hydrazide (4-ABAH; a specific inhibitor

Table 1. Effect of the inhibitors of the production of ROS/RHS and their scavengers on the parameters characterizing the chemical transformation of gallocyanine in a suspension of neutrophils activated by fMLP or PMA

Agonist	Parameter	SOD, 1.6 μM	CP, 1.1 μM	4-ABAH, 100 μM	Taurine, 3.2 mM	Mannitol, 10 mM
fMLP, 0.5 μM	*v*, % of control	7 ± 5*	5 ± 4*	102 ± 29	98 ± 27	113 ± 20
	h_7, % of control	14 ± 4*	19 ± 18*	129 ± 30	92 ± 15	107 ± 35
PMA, 50 nM	*v*, % of control	0**	2 ± 3*	93 ± 19	105 ± 20	125 ± 20
	h_7, % of control	0**	6 ± 9*	127 ± 23	104 ± 19	110 ± 15

* $p < 0.05$.
** Complete absence of the increment in fluorescence 20 min after the activation of neutrophils.

of the catalytic activity of MPO and hence HOCl production [19]), taurine (a HOCl scavenger), and mannitol (a hydroxyl radical scavenger) had no significant effect on the oxidation of gallocyanine in the presence of activated neutrophils. However, after the addition of SOD or ceruloplasmin (CP), the values of the amplitudes of fluorescence intensity (h_7) and the reaction rate (v) were significantly lower than the control (the activation of neutrophils by fMLP or PMA without inhibitors or scavengers of ROS/RHS) or were not determined at all due to the absence of fluorescence increment 20 min after the activation of neutrophils. As known, SOD eliminates $^•O_2^-$, by catalyzing its dismutation according to reaction (2) to H_2O_2, which, as we were able to find out, does not react with gallocyanine (see above). The effect of CP is, most likely, due to the SOD activity of this protein. These results suggest that the main contribution to the oxidation of gallocyanine in a suspension of neutrophils comes from its reaction with $^•O_2^-$.

It should be noted that, in human blood, MPO is overwhelmingly concentrated in neutrophils, and only a small portion of it is in monocytes [1, 4]. As for NADPH oxidase, its various isoforms are found not only in neutrophils but almost in all other types of blood cells [2]: monocytes [21], lymphocytes [22], platelets [23], and erythrocytes [24].

Thus, the results obtained allow us to conclude that gallocyanine can act as a fluorogenic chemosensor and be used to estimate the NADPH-dependent production of superoxide anion radical, most likely, not only by neutrophils but also by other blood cells and to test the antioxidant properties of drugs designed with the aim to correct diseases associated with oxidative stress.

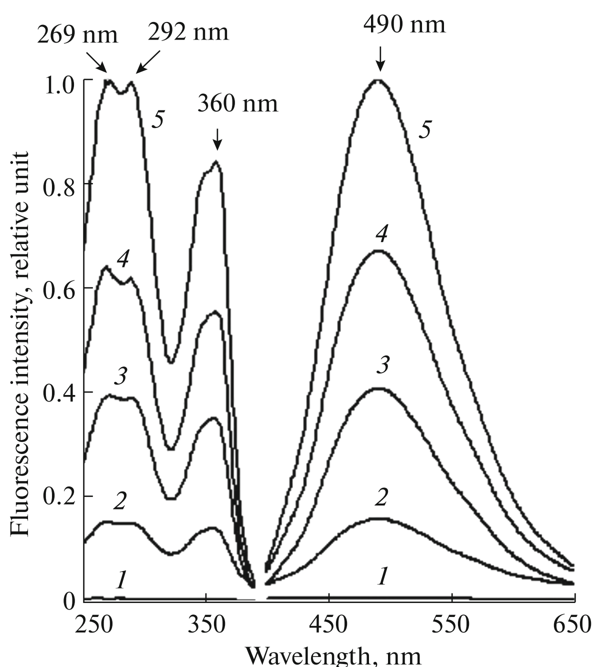

Fig. 8. Spectra of excitation and emission of gallocyanine fluorescence before and after the addition of HOCl to the gallocyanine solution. Measuring medium PBS-1, pH 7.4. Gallocyanine concentration 5 μM. The concentration of HOCl (μM): (*1*) 0; (*2*) 2.5; (*3*) 5.0; (*4*) 7.5; (*5*) 10.0. Excitation at 360 nm, emission at 490 nm.

EXPERIMENTAL

Reagents. The following reagents were used: sodium citrate, fMLP, PMA, NaOCl, gallocyanine, KO_2, xanthine oxidase, xanthine, 4-ABAH, SOD, taurine, mannitol, 2,5-dihydroxybenzoic acid (Sigma-Aldrich, United States); dextran T70 (Roth, Germany); and Histopaque (Nycomed, Norway). Other reagents were from Reakhim (Russia) and Belmedpreparaty (Belarus). CP was isolated from the citrate-stabilized blood plasma of healthy donors by ion-exchange and affinity chromatography [20].

The concentration of hypochlorite anion (OCl^-) was determined by its absorption at 290 nm and at pH 12.0 using the molar extinction coefficient ε_{290} = 350 M^{-1} cm^{-1} [25]. Considering that pK_a of HOCl is ~7.5 [25] and that, at physiological pH values, about half of the acid is in the molecular form and the rest of the acid in the form of anion, by HOCl we meant the $HOCl/OCl^-$ mixture present in the medium being examined.

The phosphate-buffered saline containing 10 mM Na_2HPO_4/KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.4 (PBS-1) or 10 mM Na_2HPO_4/NaH_2PO_4, pH 7.4 (PBS-2) served as a buffer.

Absorption spectra were recorded on double-beam spectrophotometers PB 2201 (Solar, Belarus) and Cary-50 (Varian, United States).

Fluorescence measurements were performed on a SM 2203 spectrofluorimeter (Solar, Belarus).

Isolation of neutrophils was carried out by centrifugation in a density gradient of Histopaque from the blood of healthy donors as described in [26]. The resulting sediment of neutrophils was washed with PBS-1 containing 2 mg/mL of D-glucose at 4°C and immediately used in the experiment.

Production of ROS and RHS by neutrophils was estimated by fluorescence using gallocyanine. Gallocyanine (5 μM), fMLP (0.5 μM), PMA (50 nM), and the inhibitors of production of ROS/RHS or their scavengers (SOD, 4-ABAH, CP, taurine, mannitol) were added to neutrophils (10^6 cells/mL) in PBS-1 containing 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$. The registration was performed at 37°C under continuous stirring of the sample. The production of $^{\bullet}O_2^-$ in the xanthine/xanthine oxidase system was recorded in PBS-2 at 25°C; the concentration of xanthine was 1 mM, that of xanthine oxidase was 0.52 μM, and that of gallocyanine was 5 μM. For the fluorescence registration, the excitation and emission wavelengths were empirically chosen to be 360 and 490 nm, respectively. The chemical conversion of the dye was characterized using the following parameters (Fig. 4): the tangent of the slope angle of the initial linear portion of the kinetic curve of fluorescence intensity changes (v), which characterizes the reaction rate, and the fluorescence intensity of the sample as compared with the background level 7 (h_7) or 20 (h_{20}) min after the initiation of neutrophil activation, which reflects the amount of accumulated fluorescent reaction products.

Mass spectra were recorded on an Ultraflex II TOF/TOF mass spectrometer (Bruker Daltonics, Germany) equipped with a UV laser (Nd) in the positive ion mode using a reflectron. The lag time of the analyzer was 200 ns. The voltage was 25.01 kV at the electrode of the accelerator, 21.71 kV at the accumulating electrode, and 10.01 kV at the focusing lens. The parameters of the mass spectrometer were optimized for the m/z range 50–1000. The signal was accumulated as a signal averaged over 200 single impulses; the power of laser radiation was constant at the threshold level to increase the resolution. 2,5-Dihydroxybenzoic acid was used as a matrix.

Statistical and graphical processing of data was performed using the software package OriginPro 2016. The data are presented as the mean ± standard error of the mean. The differences were calculated using the Student's t-test and were considered statistically significant at $p < 0.05$. Typical kinetic curves are an invariant of three to five independent experiments.

FUNDING

The study was supported by the Russian Foundation for Basic Research (project 20-515-00006) and Belarusian Foundation for Basic Research (project B20R-215) CP was purified with financial support from the MD-1901.2020.4 grant.

COMPLIANCE WITH ETHICAL STANDARDS

The paper does not contain any study involving animal participants performed by any of the authors.

All procedures performed using human participants comply with ethical standards approved by the institutional and/or national committees on research ethics and the Helsinki Declaration of 1964 and its ensuing alterations or with comparable ethical standards.

From each of human participants of the study, voluntary informed consent was received.

Conflict of Interests

The authors declare that there is no conflict of interest.

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Translated by S. Sidorova