Reaction of ferricytochrome *c* with the iron nitrosyl complex $\{Fe_2[S(CH_2)_2NH_3]_2(NO)_4\}SO_4 \cdot 2.5H_2O$

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By an example of cysteamine iron nitrosyl complex $\{Fe_2[S(CH_2)_2NH_3]_2(NO)_4\}SO_4 \cdot 2.5H_2O$ (CAC) it was shown for the first time that the NO donor hydrolysis in the presence of ferricytochrome *c* (cyt c^{3+}) affords the iron nitrosyl complex NO—cyt c^{3+}. It was found that cyt c^{3+} can serve as a depot for NO evolved during the hydrolysis of CAC. In the presence of CAC, the rate of NO—cyt c^{3+} complex decomposition to NO and cyt c^{3+} depends on the molar ratio [cyt c^{3+}] : [CAC] and at [cyt c^{3+}] : [CAC] = 0.3 it was found to be lower than that in decomposition of CAC in the absence of cyt c^{3+}. As a result, the total NO evolving process becomes 5.6 times more prolonged. The number of NO groups evolved from CAC can be determined by the reaction of CAC with cyt c^{3+} in the presence of ferricyanide: at most one NO group is evolved to a solution in the spontaneous hydrolysis of CAC (pH 7.0), and no less than three of them are evolved from oxidized CAC.

Key words: cytochrome *c*, ferricytochrome *c*, iron nitrosyl complexes, NO donors, cytochrome nitrosyl complexes.

Lately, an intense search for new exogenous nitrogen monoxide (NO) donors are aimed at creating new methods for the generation of NO as one of necessary universal regulators of cellular metabolism functions.[1] The use of medications producing NO under physiological conditions in clinics has essential disadvantages caused by a nitrate tolerance, the necessity of additional activation, *etc*. The models of active sites of non-heme iron-sulfur proteins, *viz*., iron nitrosyl complexes (INC) with functional sulfur-containing ligands synthesized at the Institute of Problems of Chemical Physics of the Russian Acadeny of Sciences,[2,3] spontaneously generate NO in aqueous solution as a result of hydrolysis, so they are promising new generation prodrugs.[4,5] It is of interest to study the mechanisms of action of these compounds as the NO donors on the function of heme proteins with the aim of their possible use as vasodilating, antihypertensive, and antiaggregatory medications.

It was previously found that INC nitrosylate hemoglobin (Hb).[2,3] The HbNO complex that formed provides the stabilization of NO (because the lifetime of free NO in cells comprises seconds)[6] and determines the prolongation of the INC action as the NO donors. Besides Hb, cytochrome *c* (cyt c) can form sufficiently stable nitrosyl complexes regardless of the fact that the Fe atom in heme cyt c is completely coordinated by four N atoms of porphyrin and by amino acids histidine-18 and methionine-80.[7]

The reactions of NO with cytochrome *c* are of great significance. The NO—cyt c^{3+} complex is the temporary depot for nitrogen oxide in organism and can evolve NO under certain conditions. It is known that cyt c is an essential component of the respiratory chain, and, being the regulator of oxidative processes in cells, it is of great importance in the processes of cellular energy production.[7] It accomplishes the electron transfer in the terminal reaction of the mitochondrial respiratory chain. A blockage of this process disrupts the normal energy supply of the cell. Cytochrome *c* takes an active part in the transport processes through a mitochondrial inner membrane. Ferri and ferro forms of cyt c (cyt c^{3+} and cyt c^{2+}, respectively) are able to selectively combine different cations and anions, ATP and ADP,[7] and to regulate their transport in mitochondria. The ion transport function of cyt c is significant for the maintenance of the physiological, *i.e*. electrochemical, potential of a connecting membrane and for the related ATP formation process. It was found that the concentration of cyt c varies for different diseases: it is reduced during chronic hypochromic anemia, malignant neoplasms, and acute hemorrhage. Besides, it is known that cyt c nitrosyl complexes are photosensitive and decompose under the visible light radiation. This has the therapeutic effect. Thus, the laser emission can effect on the peroxidase activity of cyt c and apoptosis.[7] Thus, it is obvious from stated above that the formation of cyt c nitrosyl complexes can affect the metabolism processes

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within a wide range. This can determine different properties of INC as drugs if they are included in the cyt c metabolism as the NO donors. The aim of the present study is to research the reaction of a new INC representative, the water-soluble cationic cysteamine nitrosyl complex $\{Fe_2[S(CH_2)_2NH_3]_2(NO)_4\}SO_4 \cdot 2.5H_2O$ (CAC)[8] with cyt c^{3+}. So far, no NO donor was investigated in the cyt c nitrosylation.

Experimental

Horse heart cytochrome *c* (Serva, Germany), Sephadex G-25 (Pharmacia, Sweden), $Na_2HPO_4 \cdot 6H_2O$ and $NaH_2PO_4 \cdot H_2O$ (MP Biomedicals, Germany), $Na_2[Fe(CN)_5NO] \cdot 2H_2O$ and $NaNO_2$ (Aldrich, USA), $K_3Fe(CN)_6$ (CP, Khimmed, Russia) were used. Water was distilled in a distiller Bi/Duplex (Germany).

Absorption spectra were recorded on a Specord M-40 spectrophotometer equipped with a computer interface for the spectra registration and a temperature-controlled cell.

All operations were carried out under high purity nitrogen that was additionally purified on a column with a chromium nickel catalyst. In order to transfer a buffer and working solutions into the nitrogen atmosphere, the nitrogen was bubbled through them for 30 min with stirring. Hereinafter, we will call such solutions anaerobic. All receptacles and quartz cells used were sealed with Rubber Septa rubber sleeve stoppers (Sigma) that allowed the injection of gas or required components through a needle. The solutions were transferred from one receptacle to another either using soldered needled syringes or under an excess nitrogen pressure with two needles connected by a Teflon capillary. The excess pressure was dissipated through another needle connected with the Teflon capillary immersed into water. Nitrogen was bubbled through the cells and tubes with the reagents with volumes of 4 and 10 mL, respectively, through the needles for 30 min.

Bis(cysteaminylthio)tetranitrosyl diiron (CAC) was synthesized according to the previously described procedure.[8] An elemental analysis of the polycrystalline powder obtained was performed at the Analytical Center of the Institute of Problems of Chemical Physics of the Russian Academy of Sciences. Found (%): C, 8.53; H, 2.77; N, 15.70; S, 17.71. $C_4H_{19}Fe_2N_6O_{10.5}S_3$. Calculated (%): C, 9.10; H, 3.60; Fe, 21.25; N, 15.93; O, 31.87; S, 18.27.

Cyt c^{3+} solution was prepared from a commercial cyt c^{3+} specimen containing 10% cyt c^{2+}. In all steps of the preparation of cyt c^{3+} and in all experiments with it, a 0.05 *M* phosphate buffer (pH 7.0) was used. Commercial cytochrome *c* (9 mg) was dissolved in a 0.05 *M* phosphate buffer (pH 6.5) (3 mL) with stirring, and then a potassium ferricyanide solution ($2 \cdot 10^{-2}$ mol L^{-1}) in a 0.05 *M* phosphate buffer (pH 6.5) (0.1 mL) was added. The oxidation of cyt c^{3+} was carried out with stirring during 10 min at 10 °C. The absorption spectrum of the solution aliquot was recorded and the obtaining of cyt c^{3+} was confirmed by the fact that the absorption maximum and the extinction coefficient are consistent with the literature data.[9] An excess of potassium ferricyanide was removed on a column (21×5 cm, Sephadex G-25) through which three column volumes (150 mL) of a phosphate buffer, pH 7.0, were passed beforehand; 5.8 mL cyt c^{3+} ($9.9 \cdot 10^{-5}$ mol L^{-1}) were obtained from the column outlet. The cyt c^{3+} was stored frozen in ball form under liquid nitrogen. It was defrosted in 5 mL tubes under a nitrogen stream before use. The nativity and homogeneity of the cyt c^{3+} were confirmed by the fact that the extinction coefficients of all absorption maxima are consistent with the literature data. In those cases when the reaction mixtures were investigated in the presence of ferricyanide, commercial cyt c^{3+} was not pre-oxidized. The ferricyanide solution was put into a cell with cyt c^{3+}, and the absorption spectrum was recorded for checking the completeness of the oxidation.

Reaction of NO with cyt c^{3+}. Cyt c^{3+} (2.5 mL), pH 7.0, prepared as was described above was put into an anaerobic 4 mL sample cell (optical path length was 1 cm). The absorption spectrum was recorded. Then NO was injected into the cell for 3 min, and the cyt c^{3+} solution was shaken. The absorption spectrum of the solution was recorded and the obtaining of NO—cyt c^{3+} was confirmed.

Hydrolysis of CAC at pH 7.0. A 0.05 *M* phosphate buffer (2 mL), pH 7.0, was put into an anaerobic 4 mL sample cell (optical path length was 1 cm). A 0.05 *M* anaerobic phosphate buffer, pH 7.0, was added to the CAC complex in a tube filled with nitrogen to obtain the complex solution with a concentration of $6 \cdot 10^{-4}$ mol L^{-1}. Then the solution was stirred for 15 min untill the complex was completely dissolved. The solution obtained (1 mL) was injected into the sample cell. A reference cell contained 3 mL of the anaerobic buffer. The final CAC concentration was $2 \cdot 10^{-4}$ mol L^{-1}. The absorption spectra were recorded within 30 s after the CAC injection and then in 15 min intervals for 3 h.

Hydrolysis of CAC at pH 7.0 (in the presence of ferricyanide). A 0.05 *M* phosphate buffer (1.7 mL), pH 7.0, and the obtained CAC complex solution (1 mL) were put into an anaerobic 4 mL sample cell (optical path length was 1 cm). A reference cell contained 2.7 mL of the anaerobic buffer. The final CAC concentration was $2 \cdot 10^{-4}$ mol L^{-1}. The absorption spectrum of the solution was recorded. Then a potassium ferricyanide solution with a concentration of $2 \cdot 10^{-2}$ mol L^{-1} (by 0.3 mL) was added to the sample cell and the reference cell. The absorption spectra were recorded within 30 s after the injection of the potassium ferricyanide and then in 2—3 min intervals for 1.5 h.

Kinetics of the reaction of cyt c^{3+} with CAC. The cyt c^{3+} solution (2 mL) earlier prepared was injected into an anaerobic 4 mL sample cell (optical path length was 1 cm) to the concentration of $3 \cdot 10^{-5}$ mol L^{-1}. Then the CAC solution ($6 \cdot 10^{-4}$ mol L^{-1}, by 1 mL) was added to the cell with cyt c^{3+} and to the reference cell containing 2 mL of the anaerobic buffer. The final CAC concentration was $2 \cdot 10^{-4}$ mol L^{-1}. The absorption spectra were recorded within 30 s after CAC injection and then in 15 min intervals for 1.5 h.

Kinetics of the reaction of cyt c^{3+} with CAC in the presence of potassium ferricyanide. In studying the kinetics of the reaction of cyt c^{3+} with CAC in the presence of potassium ferricyanide, the molar ratios [cyt c^{3+}] : [CAC] = 0.3, 4.3, and 7.85 were used. For that, into a sample cell we added a solution of commercial cyt c^{3+} in a phosphate buffer (1.7, 2.0, and 2.36 mL), pH 7.0, at concentrations of 10^{-4}, 10^{-4}, and $2 \cdot 10^{-4}$ mol L^{-1}, respectively, a potassium ferricyanide solution (0.3 mL) at a concentration of $4 \cdot 10^{-2}$ mol L^{-1}, and an amount of the buffer such that, after the addition of the CAC solution, the volume of the reaction solution was 3.0 mL. The absorption spectrum of the solution was recorded and the obtaining of cyt c^{3+} was confirmed.

A reference cell contained potassium ferricyanide (0.3 mL, $4 \cdot 10^{-2}$ mol L^{-1}) and an anaerobic buffer (1.7, 2.62, and 2.6 mL, respectively). The reaction was initiated by the simultaneous addition into the sample and the reference cells of a CAC solution (1.0, 0.08, and 0.1 mL, respectively; $6 \cdot 10^{-4}$ mol L^{-1}). Then difference absorption spectra were recorded in particular time intervals. The spectra were recorded until the complete transformation of cyt c^{3+} into the NO—cyt c^{3+} complex when the spectrum ceased to change.

Determination of the amount of the NO—cyt c^{3+} complex. In order to determine the concentration of the formed complex, the absorption spectrum of the reaction mixture containing cyt c^{3+} and NO—cyt c^{3+} was resolved into the component spectra of cyt c^{3+} and NO—cyt c^{3+} by the computer processing with the MathCad program as has been described earlier.[2]

Results and Discussion

Let us consider principal properties of nitrosylated cytochromes. The structure investigations[10—13] of cyt c showed that the heme group serves as a prosthetic group around which a polypeptide chain folds; the number of amino-acid residues of cytochrome *c* (104) is enough for the formation of a polypeptide shell around the heme. Both in the oxidized and reduced protein forms, besides the main porphyrin ligands in four coordination positions of the Fe atom, the fifth and sixth positions are occupied by methionine-80 (Met80) and histidine-18 (Fig. 1).[14] The redox potential of cyt c at 25 °C and pH 7.0 with respect to the hydrogen electrode is 0.254 V.[15] In spite of the fact that all coordination positions in the heme of cyt c are occupied, it can react with NO. The reaction of NO with the heme leads to the cleavage of the bond between the methionine residue and the iron ion followed by the replacement of NO as the sixth ligand with methionine.

The reversible formation of the adduct NO—cyt c^{3+} occurs:

$$\text{NO} + \text{cyt } c^{3+} \rightleftharpoons \text{NO—cyt } c^{3+}. \qquad (1)$$

For this reaction,[9,16] the association constant (k_{as}) is $7.2 \cdot 10^2$ L mol^{-1} s^{-1}, the dissociation constant (k_{dis}) is $4.4 \cdot 10^{-2}$ s^{-1}, and the equilibrium constant (K_{eq}) is $1.6 \cdot 10^4$ L mol^{-1}.

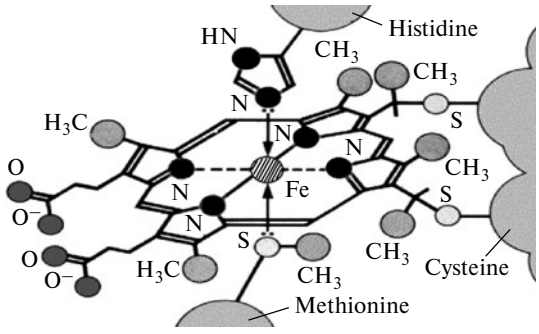

Fig. 1. Structure of cyt c heme.[14]

The lower reaction rates and the equilibrium constants compared to those in the case of Hb are attributed to the energy consumption for the substitution of the sixth ligand. Indeed, cyt c in which Met80 is alkylated and, hence, the heme iron is five-coordinate and has the NO affinity three orders of magnitude higher ($2 \cdot 10^7$ L mol^{-1}) than that of the native protein.[9] In the reaction of cyt c^{3+} with NO the changes in the absorption spectrum are observed:[17] the spectrum of the NO—cyt c^{3+} complex has maxima at $\lambda = 415$ ($\varepsilon = 1.45 \cdot 10^5$ L mol^{-1} cm^{-1}), 527 ($\varepsilon = 1.0 \cdot 10^4$ L mol^{-1} cm^{-1}), and 560 nm.[16]

Since the aim of the present work was to study the reaction of CAC with cyt c^{3+} we obtained preparative amounts of cyt c^{3+} from commercial cyt c. The commercial cyt c consisting of a mixture of cyt c^{3+} (90%) and cyt c^{2+} (10%) was additionally oxidized with potassium ferricyanide. Since the reaction of cyt c^{3+} with NO at pH 7.0 causes changes in the absorption spectrum, it was possible to measure the kinetics and the rate constant of the reaction of CAC with cyt c^{3+} at pH 7.0 based on the amount of the formed NO—cyt c^{3+}.

Although individual cyt c^{3+} does not react with oxygen, we carried out all the works with CAC and all following reactions of CAC with cyt c^{3+} under the nitrogen atmosphere, since NO readily reacts with O_2 to form nitrogen oxides; the rate constant of this reaction is $2 \cdot 10^6$ $(L\ mol^{-1})^2$ s^{-1}.[18]

First, the stability of CAC at pH 7.0 was estimated. For that, changes in the absorption spectrum of the solu-

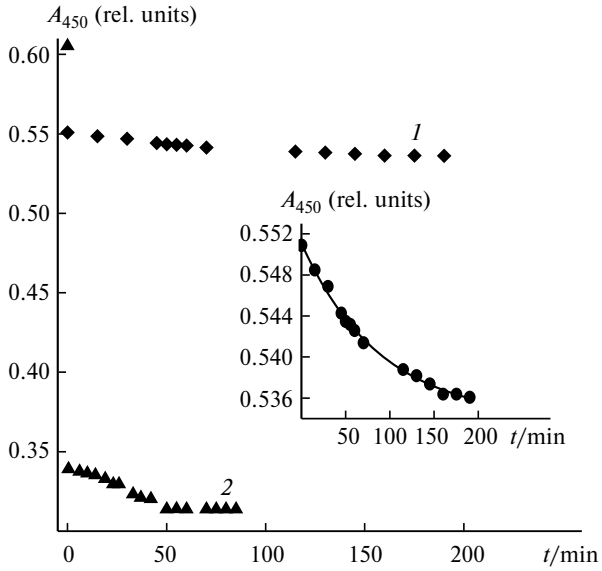

Fig. 2. Kinetics of the CAC ($2 \cdot 10^{-4}$ mol L^{-1}) hydrolysis in the absence (*1*) and in the presence of ferricyanide ($4 \cdot 10^{-3}$ mol L^{-1}) (*2*). The inset shows the kinetics of the CAC hydrolysis in the absence of ferricyanide after the computer processing of the curve *1*. The reaction conditions: 23 °C, solvent is 0.05 *M* phosphate buffer, pH 7.0.

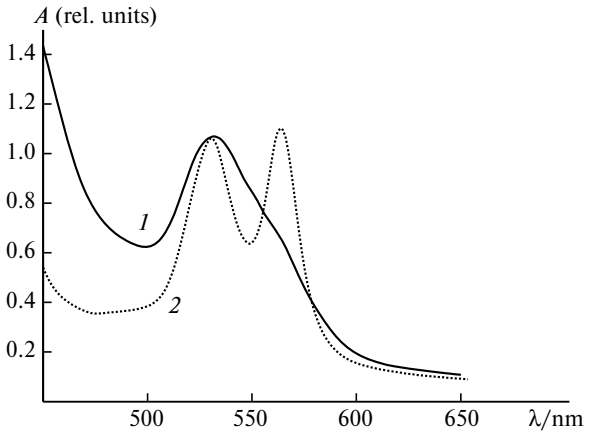

Fig. 3. Absorption spectra of cyt c^{3+} ($9.7 \cdot 10^{-5}$ mol L^{-1}) in a 0.05 *M* phosphate buffer (*1*) and of the NO—cyt c^{3+} complex (*2*) obtained after equilibration of this cyt c^{3+} solution with gaseous NO. Conditions: pH 7.0, 23 °C, 760 Torr NO.

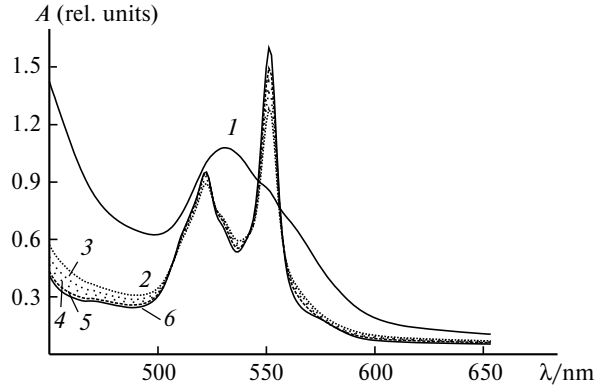

Fig. 4. Time-resolved difference absorption spectra for the reaction of CAC ($2 \cdot 10^{-4}$ mol L^{-1}) with cyt c^{3+} ($9.8 \cdot 10^{-5}$ mol L^{-1}): the spectrum of the starting cyt c^{3+} (*1*) and the spectra recorded within 0.5 (*2*), 10 (*3*), 20 (*4*), 60 (*5*), and 70 min (*6*) after the beginning of the reaction. The reaction conditions are in the caption for Fig. 2.

tion of CAC in the buffer (pH 7.0) were recorded in a range λ = 450—650 nm. Then the kinetic curves of the absorbance (*A*) at λ = 450 nm were plotted (Fig. 2). The CAC proved to be rather stable at pH 7.0 (see Fig. 2, curve *1*). The results of the computer processing of the kinetic data with the MathCad program are represented in the inset in Fig. 2. The kinetic curve corresponds to the first-order reaction, its rate constant is $(1.8 \pm 0.2) \cdot 10^{-4}$ s^{-1}.

The absorption spectra of cyt c^{3+} with a maximum at λ = 531 nm and of NO—cyt c^{3+} with two maxima at λ = 530 and 562 nm are presented in Fig. 3. It was found that even after 24 h the absorption spectrum of NO—cyt c^{3+} (see Fig. 3) remained unchanged because of an excess of NO.

Then we studied the reaction of CAC with cyt c^{3+}. This reaction should consist of at least two steps: the hydrolysis of CAC and the following reversible reaction of the evolved NO with cyt c^{3+} (see Eq. (1)).

In order to study this reaction the components were incubated (see the Experimental) under anaerobic conditions (nitrogen atmosphere) at 23 °C. We recorded the time-resolved absorption difference spectra of the reaction mixture and a solution in a sample cell containing CAC since CAC has absorption in the visible region. The measurements were stopped when the absorption spectrum ceased to change.

The results of the study of the reaction of CAC with cyt c^{3+} are presented in Fig. 4. Spectrum *1* corresponds to the starting cyt c^{3+}. It is obvious that immediately after the addition of CAC into the cell wih cyt c^{3+}, the spectrum of cyt c^{2+} with two maxima at λ = 521 and 551 nm appears.[9,16] This is due to the fact that the redox potential of CAC at pH 7.0 relative to the hydrogen electrode is about −0.8 V by analogy with the compounds with similar structure,[19] and the redox potential of cyt c^{3+} under the same conditions is 0.254 V. Therefore the reduction occurs readily and at such a high redox potential difference, cyt c^{3+} is completely transformed into cyt c^{2+}. Since cyt c^{2+} very slowly reacts with NO at pH 7.0,[20] the formation of NO—cyt c^{2+} (absorption spectrum maxima at λ = 541 and 565 nm)[9,16] is not observed (see Fig. 4). The small changes in the spectrum are due to a slight increase in the cyt c^{2+} concentration in time.

To prevent cyt c^{3+} from the reduction with CAC the reaction was carried out in the presence of ferricyanide. Different molar ratios [cyt c^{3+}] : [CAC] (0.3, 4.3, 7.85) were used, the ferricyanide concentration was $4 \cdot 10^{-3}$ mol L^{-1}. This experiment performance is justified because CAC under the metabolism conditions, for example if used as a medication, enters the medium with many metabolites (such as all forms of cytochromes, methemoglobin, enzymes of the first and the second sections of the respiratory chain) with much more positive redox potentials than that of CAC, and the latter will certainly be reduced with these substances. The cyt c^{3+} and ferricyanide were placed into a sample cell, the buffer and ferricyanide were placed into a reference cell, and the absorption spectrum was recorded. The reaction was started by the simultaneous addition of CAC into the sample and the reference cells. The results are presented in Fig. 5. Spectrum *1* is the absorption spectrum of the starting cyt c^{3+}. Spectrum *2* recorded in 1 min after the addition of CAC is the absorption spectrum of NO—cyt c^{3+} with two maxima at λ = 530 and 562 nm. After the resolution of the spectrum it was found that the concentration of the NO—cyt c^{3+} formed was 60 μmol L^{-1}. Since the CAC concentration was 0.2 mmol L^{-1} we may conclude that in this experiment 0.3 equiv. of NO was evolved from CAC (see Fig. 5). The absorption spectra in this entry were recorded during 4 h. The spectrum changed insignificantly, and the concentration of NO—cyt c^{3+} slightly decreased. The rapid

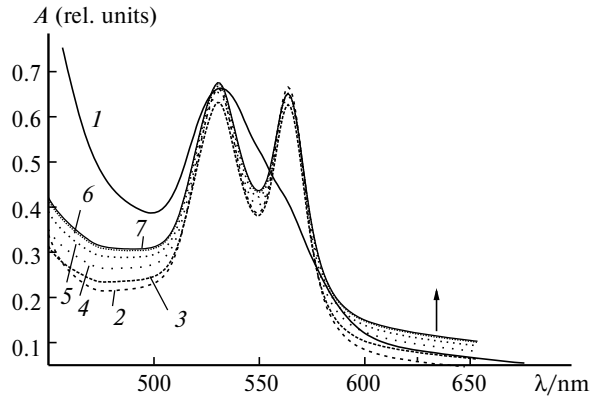

Fig. 5. Time-resolved difference absorption spectra for the reaction of CAC ($2 \cdot 10^{-4}$ mol L^{-1}) with cyt c^{3+} ($6 \cdot 10^{-5}$ mol L^{-1}) in the presence of ferricyanide ($4 \cdot 10^{-3}$ mol L^{-1}): the spectrum of the starting cyt c^{3+} (*1*) and the spectra recorded within 1 (*2*), 15 (*3*), 45 (*4*), 130 (*5*), 220 (*6*), and 235 min (*7*) after the beginning of the reaction. The reaction conditions are in the caption for Fig. 2.

formation of NO was confirmed by the control experiment that showed a prompt change in the spectrum after the addition of ferricyanide to the CAC solution virtually during the mixing time. It can be seen from Fig. 2 (curve *2*) that the absorbance at $\lambda = 450$ nm is reduced almost two times and then the color slowly changed. The rapid formation of NO—cyt c^{3+} observed in the reaction of CAC with cyt c^{3+} under these conditions is evidence of the release of the NO groups to the solution during the oxidation of CAC.

The subsequent experiments on the reaction of CAC with cyt c^{3+} in the presence of ferricyanide were carried out in the presence of an excess of cyt c^{3+} relative to CAC at the molar ratios [cyt c^{3+}] : [CAC] = 0.3, 4.3, and 7.85. The kinetics of the reaction rate was simultaneously

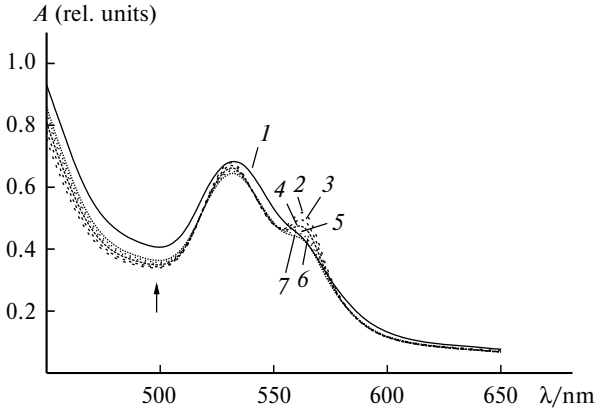

Fig. 6. Time-resolved difference absorption spectra for the reaction of CAC ($1.5 \cdot 10^{-5}$ mol L^{-1}) with cyt c^{3+} ($6 \cdot 10^{-5}$ mol L^{-1}) in the presence of ferricyanide ($4 \cdot 10^{-3}$ mol L^{-1}): the spectrum of the starting cyt c^{3+} (*1*) and the spectra recorded within 1 (*2*), 15 (*3*), 36 (*4*), 66 (*5*), 96 (*6*), and 156 min (*7*) after the beginning of the reaction. The reaction conditions are in the caption for Fig. 2.

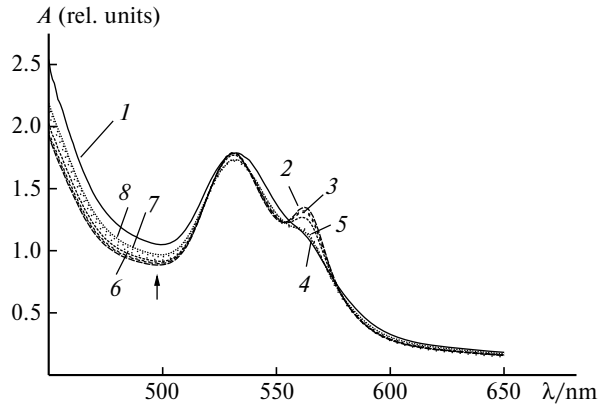

Fig. 7. Time-resolved difference absorption spectra for the reaction of CAC ($2 \cdot 10^{-5}$ mol L^{-1}) with cyt c^{3+} ($1.57 \cdot 10^{-4}$ mol L^{-1}) in the presence of ferricyanide ($4 \cdot 10^{-3}$ mol L^{-1}): the spectrum of the starting cyt c^{3+} (*1*) and the spectra recorded within 0.5 (*2*), 5 (*3*), 7.5 (*4*), 19 (*5*), 64 (*6*), 94 (*7*), and 124 min (*8*) after the beginning of the reaction. The reaction conditions are in the caption for Fig. 2.

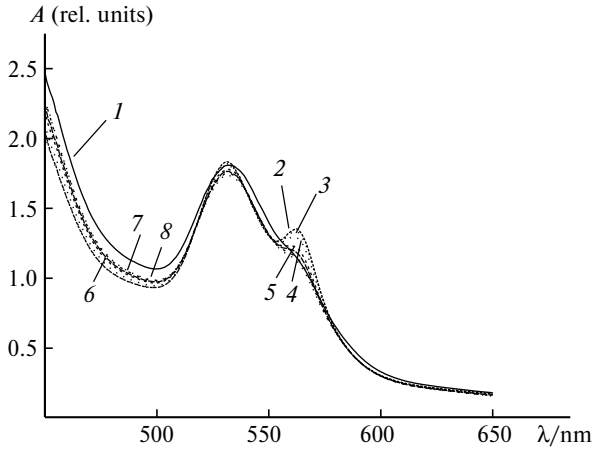

Fig. 8. Time-resolved difference absorption spectra for the reaction of CAC ($2 \cdot 10^{-5}$ mol L^{-1}) with ferricyanide ($4 \cdot 10^{-3}$ mol L^{-1}) and cyt c^{3+} ($1.8 \cdot 10^{-4}$ mol L^{-1}) after the CAC hydrolysis for 3 h at 20 °C: the spectrum of the starting cyt c^{3+} (*1*) and the spectra recorded within 1 (*2*), 3 (*3*), 6 (*4*), 13 (*5*), 43 (*6*), 133 (*7*), and 193 min (*8*) after the beginning of the reaction of CAC with cyt c^{3+}. The reaction conditions are in the caption for Fig. 2.

determined in these experiments. As in the previous experiment on the reaction of CAC with cyt c^{3+} the changes in the difference spectra with time were measured (Figs 6—8). Then each absorption spectrum was decomposed into components by computer processing with the MathCad program, the concentration of cyt c^{3+} and the conversion were determined. It was found from Fig. 6 after the decomposition of the spectrum *2* into components that NO—cyt c^{3+} at a concentration of 31.4 μmol L^{-1} was formed during 1 min. Since the CAC concentration was 0.015 mmol L^{-1} we may conclude that

in this experiment 2.1 equiv. of NO was evolved from CAC (see Fig. 6).

In the next experiment (see Fig. 7) the molar ratio [cyt c^{3+}] : [CAC] was increased to 7.85. As in the previous entry the absorption spectrum was decomposed into components. In this case after 30 s from the beginning of the reaction (see Fig. 7, spectrum *2*), the concentration of NO—cyt c^{3+} was 61.6 μmol L^{-1}. In this entry the CAC concentration was 0.02 mmol L^{-1}; therefore, 3.08 NO groups evolved to the solution. As in the entry in Fig. 6, the NO—cyt c^{3+} is rapidly formed because of the rapid conversion of CAC (see Fig. 7, curve *2*) with the release of NO into the solution during the oxidation with ferricyanide, as follows from our experiments.

It was interesting to determine the number of NO groups that can be evolved by CAC in the spontaneous hydrolysis at pH 7. Such data were not found in the literature. Since, as it is evident from the experiments described above, during the oxidation of CAC with ferricyanide, the NO releases into solution, the reaction of CAC with cyt c^{3+} can be used as a test for the number of NO groups evolved in the hydrolysis of CAC by a certain moment. The hydrolysis of CAC was carried out at pH 7.0 for 3 h under the same conditions as in the entry presented in Fig. 2, after that the formed NO was removed from the solution by bubbling, then the cyt c^{3+} and ferricyanide were added in such concentrations that it could be determined that three NO groups were evolved from CAC (see Fig. 7). Then, as in the previous entries, the changes in the absorption spectrum were recorded (see Fig. 8). In this case, after 1 min from the reaction start (see Fig. 8, spectrum *2*) the concentration of the formed NO—cyt c^{3+} was 53 μmol L^{-1}. In this entry the CAC concentration was 0.02 mmol L^{-1}; therefore, 2.65 NO groups, *i.e.* 0.43 NO groups less compared to the entry in Fig. 7, evolved to the solution. This is the evidence that in this case, 0.43 NO groups evolved to the solution during the spontaneous hydrolysis at pH 7.

Thus, the number of NO groups that could be evolved from CAC can be determined spectrophotometrically by the reaction of CAC with cyt c^{3+} in the presence of ferricyanide. It was found that during the spontaneous CAC hydrolysis at pH 7.0 at most one NO group, and no less than three NO groups evolve to the solution from the oxidized CAC.

In the entries presented in Figs 5—7 an interesting phenomenon, *viz.* the slow decomposition of nitrosylated cytochromes, was observed. This is the reliable experimental fact. It was interesting to compare the rate constants of these processes with the rate constant of the decomposition of CAC in the absence of cyt c^{3+} (see Fig. 2) that is equal to $(1.8\pm0.8)\cdot10^{-4}$ s^{-1}. On the basis of the calculated concentrations of the formed NO—cyt c^{3+} we plotted kinetic curves (Fig. 9, *a*—*c*). The rate constants were obtained by the analysis of the kinetic curves for the

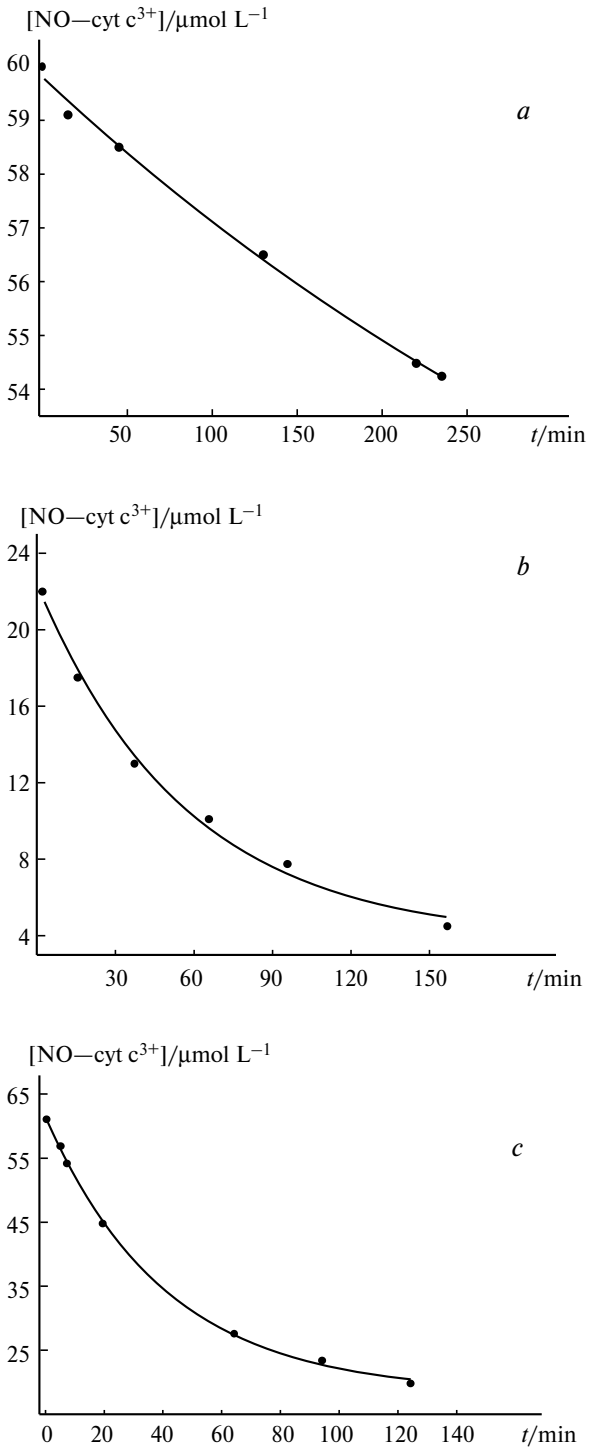

Fig. 9. Kinetics of the formation of NO—cyt c^{3+} in the presence of ferricyanide ($4\cdot10^{-3}$ mol L^{-1}) in the reaction of CAC ($2\cdot10^{-4}$ mol L^{-1}) with cyt c^{3+} ($6\cdot10^{-5}$ mol L^{-1}) according to the data from Fig. 5 (*a*); CAC ($1.5\cdot10^{-5}$ mol L^{-1}) with cyt c^{3+} ($6.5\cdot10^{-5}$ mol L^{-1}) according to the data from Fig. 6 (*b*); CAC ($2\cdot10^{-5}$ mol L^{-1}) with cyt c^{3+} ($1.57\cdot10^{-4}$ mol L^{-1}) according to the data from Fig. 7 (*c*). Theoretical curves are plotted using the equation $y(t) = ae^{-kt}$, circles denote the experimental data.

Table 1. Observed rate of decomposition of the NO—cyt c^{3+} complex (see Figs 5—7)

Figure	[CAC] /mmol L^{-1}	[cyt c^{3+}] /μmol L^{-1}	[cyt c^{3+}] : [CAC]*	[NO—cyt c^{3+}] /μmol L^{-1}	Number of NO groups	k**/s^{-1}
5	0.200	60	0.30	60.0	0.30	$(3.3\pm0.3)\cdot10^{-5}$
6	0.015	65	4.30	31.4	2.10	$(2.9\pm0.3)\cdot10^{-4}$
7	0.020	157	7.85	61.6	3.08	$(4.0\pm0.4)\cdot10^{-4}$

* Molar ratio.

** k is the effective rate constant of the total process of decomposition of NO—cyt c^{3+} in solution.

reactions of cyt c^{3+} with CAC. It was found that these curves (see Fig. 9) are well described in terms of first-order reactions. By the equation $y(t) = ae^{-kt}$ we calculated the effective first-order rate constants (k) of the studied reactions (Table 1).

As is seen from the data in Table 1, in the presence of CAC, the rate of the decomposition of the NO—cyt c^{3+} complex to NO and cyt c^{3+} depends on the molar ratio [cyt c^{3+}] : [CAC]. At [cyt c^{3+}] : [CAC] = 0.3 it is lower than the rate of the CAC decomposition in the absence of cyt c^{3+} (see Fig. 2). We can say that the total process of NO evolution becomes 5.6 times more prolonged in the presence of CAC. This conclusion is important for the CAC metabolism because, as known from the literature data,[7] cyt c^{3+} is the NO depot providing its preservation with the following release.

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