The interaction of coordinationally unsaturated 2,9-dimethylphenanthroline cobalt complex with adenine nucleotides

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The study of complex formation between adenosine triphosphate (ATP) or adenosine diphosphate (ADP) and coordinationally unsaturated cobalt complex with 2,9-dimethyl-*o*-phenanthroline (L) showed the possibility of formation of relatively stable mixed ligand complex CoL·ATP ($\log\beta = 4.45$). The latter may be formed under the oxidative phosphorylation conditions in mitochondria. The presence of ATP in the complex provides the specific inhibition of the ATPase active center.

Key words: 2,9-dimethylphenanthroline, cobalt complex, mitochondria.

The studies of biological activity of chemical catalysts *in vivo* are directed to the expansion of the views about the methods of the cell metabolism controlling and open new possibilities for the creation of biologically active compounds with catalytic properties. Previously we have studied the action of various metallocomplex redox-catalysts on the electron transport in the respiration and photosynthetic processes. In this respect cobalt complexes with phenanthroline ligand are of great interest. We have found that the coordinationally unsaturated cobalt complex with 2,9-dimethylphenanthroline of the composition $[Co(2,9\text{-}Me_2Phen)(H_2O)_2](NO_3)_2$ acts as a specific inhibitor of the oxidative phosphorylation in mitochondria.[2] We consider the elucidation of the reasons of the inhibiting action of this complex to be of importance, in particular, the studying of the possibility of inclusion of the ATP molecule into a coordination sphere of a complex and formation of the mixed complex with this bioligand under conditions of biochemical experiments with mitochondria.

Experimental

Mitochondria (MTC) of the rat liver were obtained by the described method.[3] The protein concentration was determined by the biuret reagent.[4] The rate of the oxygen absorption by mitochondria was measured polarographically.[6] The medium, in which the activity at pH 7.4 was measured, contained 0.15 *M* solution of sucrose, 75 m*M* solution of KCl, 5 m*M* solution of KH_2PO_4, 2 m*M* solution of $MgCl_2$, and 20 m*M* solution of the oxidation substrate, succinate (succinic acid). The phosphorylation rate of ADP by mitochondria was determined by the introduction of 200 m*M* of ADP. 2,4-Dinitrophenol (DNP) in the concentration of 100 m*M* was used as a separator. The commercial 2,9-dimethylphenanthroline (Chemapol) was used for the preparation of the complex. The method of the complex preparation is similar to that described previously.[6]

Spectral measurements were performed on a Specord M-400 spectrophotometer in standard quartz cuvettes with the optical length of 1 cm. The measurement error was ±1.2%. The treatment of the spectral data was performed on an IBM PC/AT personal computer according to the special program. Reagents from Serva, Sigma, and Reanal as well as domestic chemically and analytically pure grade reagents were used.

Results and Discussion

We studied the influence of the coordinationally unsaturated cobalt(II) complex of the composition $[Co(2,9\text{-}Me_2Phen)(H_2O)_2](NO_3)_2$ (CoL) and mixedligand complex with histidine $[Co(2,9\text{-}Me_2Phen)(H_2O)(His)](NO_3)_2$ (CoL·His) on the respiration and oxidative phosphorylation of mitochondria from the rat liver. As can be seen from Fig. 1, *a*, both of the complexes do not affect the respiration of the conjugated mitochondria (curves *1*, *2*). The absence of the inhibiting action of the cationic complexes with 2,9-methylphenanthroline ligand on the respiration of the conjugated MTC may be caused by the inability of the complexes to penetrate through the positively charged MTC membrane similarly to that as it was shown for tris-phenanthroline complexes of Co(II/III).[7]

On the other hand, we observed the difference in the efficiency of the action of two complexes on MTC in the presence of the separator (DNP): the CoL complex inhibited the respiration of MTC (Fig. 1, *a*, curve *3*) and oxidative phosphorylation (Fig. 1, *b*, curve *2*). It is known that DNP changes the electroconductivity of a membrane, and a charge of the external membrane becomes negative. In this case, both of the complexes can penetrate through the MTC membrane under the electric field gradient. The difference in the action of

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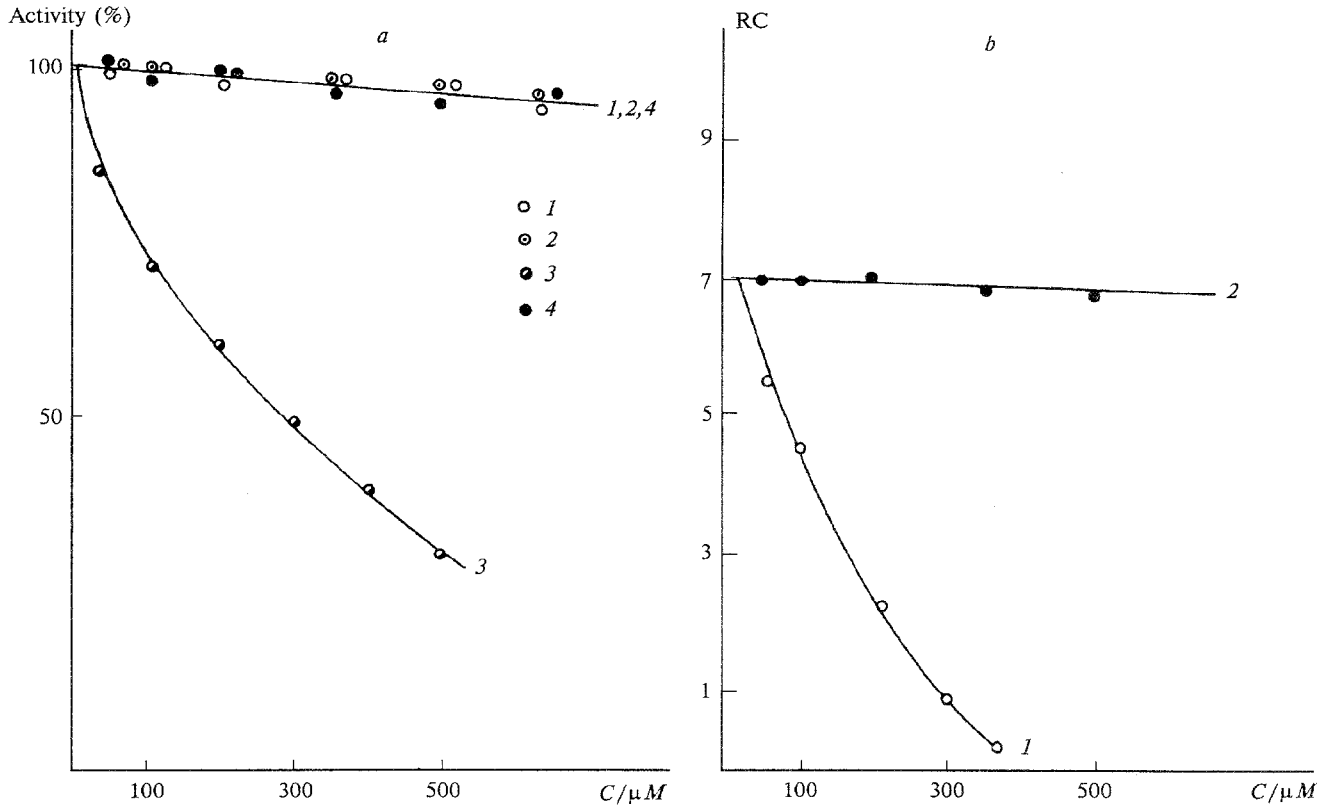

Fig. 1. Effect of *o*-phenanthroline complexes of Co(II) on respiration and oxidative phosphorylation of mitochondria.
a. Action of complexes on the MTC respiration without the separator (*1, 2*) and in the presence of $1 \cdot 10^{-4}$ *M* DNP (*3, 4*): *1, 3*, complex CoL; *2, 4*, complex CoL · His.
b. Effect of complexes on the oxidative phosphorylation (RC is respiration control): *1*, complex CoL; *2*, complex CoL · His. Conditions of measuring activity see «Materials and methods», MTC protein — 2 mg/ml.

the complexes on MTC in the presence of DNP seems to be related not to their penetrating ability, but to their different coordinational saturation and ability to coordinate additional ligands. Previously we showed the possibility to coordinate chlorine anions by the coordinationally unsaturated cobalt(II) complexes with *o*-phenanthroline and their transmembrane transfer through bilayer lipid membranes.[8]

We supposed that the ability of the CoL complex to inhibit oxidative phosphorylation in MTC is conditioned by the ability of this coordinationally unsaturated complex to include a nucleotide molecule (ATP or ADP) into its coordinational sphere and to form the stable complex [CoL · ATP (ADP)], which is the inhibitor of the ATPase enzyme. Such mixed complexes, binary CoL · ATP and triple oxygenated CoL · ATP · O_2, where L was nonsubstituted phenanthroline, were obtained in an individual form[9] and were used as inhibitors of Na^+/K^+-ATPase and ATPase of myosin.[10,11]

We have studied a possibility of the complex formation of CoL with ATP and ADP under conditions that we used in the experiments with MTC (here L is 2,9-dimethylphenanthroline). The process of a complex formation was studied spectrophotometrically by the change in the absorption spectra of the complexes in a visible range (400—800 nm) varying pH and ATP concentration. The spectra of the CoL complex in water were used as a reference.

Figure 2 presents the spectra of the phenanthroline complex CoL in different buffer systems. The main absorption maximum of the complex is in the range of 640 nm, the spectra of the complex in the phosphate buffer and buffer based on *N*-ethylmorpholine coincide with those in water (Fig. 2, *a*). Imidazole, tris(hydroxymethyl)aminomethane, and ethylenediamine hydrochloride caused the strongest effect on the character of the spectrum (Fig. 2, *b*). The complex seems to be capable of coordinating individual components of buffer systems, that results in the change of the electron spectra form. The intensity of a solution coloring depends both on the nature of a buffer used and on pH.

The studying of the dependence of the spectra of the complex CoL in water on pH showed that the increase in pH of a solution results in the increase of the absorption in the range of 640 nm (Fig. 3, *a*). Probably, hydroxyl ions existing in the solution enter into the coordinational sphere of CoL that enhances the electron density on the central metal atom. Considering the CoL

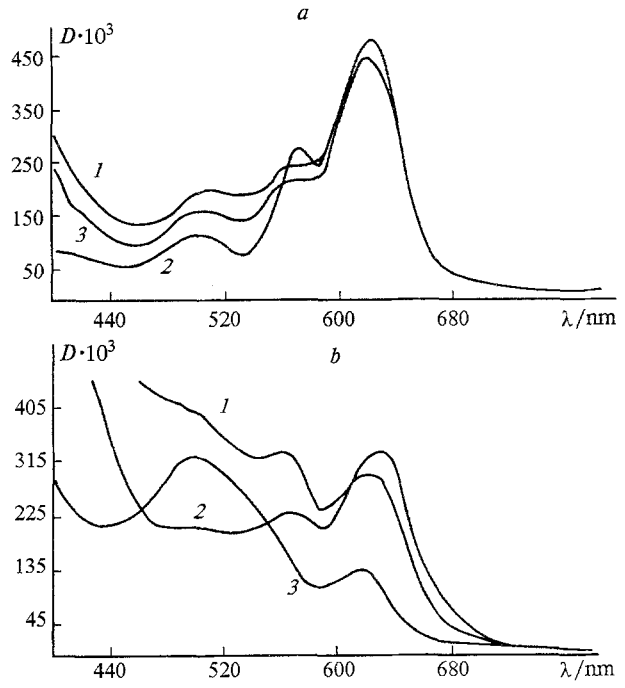

Fig. 2. Absorption spectra of the complex $[Co(2,9\text{-}Me_2phen)(H_2O)_2](NO_3)_2$ in different buffer systems.
a. 1, *N*-ethylmorpholine ($5 \cdot 10^{-3}$ *M*; pH 7.5); *2*, KH_2PO_4 ($5 \cdot 10^{-3}$ *M*; pH 7.5); *3*, H_2O. Concentration of $[Co(2,9\text{-}Me_2phen)(H_2O)_2](NO_3)_2$ is $5 \cdot 10^{-3}$ *M*.
b. 1, ethylenediamine hydrochloride ($5 \cdot 10^{-2}$ *M*; pH 9.0); *2*, tris(hydroxymethyl)aminomethane ($5 \cdot 10^{-2}$ *M*; pH 9.0); *3*, imidazole ($5 \cdot 10^{-2}$ *M*; pH 9.0).

complex to be a protonated particle, one can formally describe this process by the following equilibrium scheme:

$$CoL(OH)^- + H^+ \rightleftharpoons CoL + H_2O.$$

The protonation constant of the complex $CoL(OH)^-$ ($pK_a = 6.68 \pm 0.077$) was calculated and the distribution curves of the CoL and $CoL(OH)^-$ particles depending on pH were obtained (Fig. 3, *b*) based on the spectral data obtained by means of the program, whose algorithm is given below.

The essence of the algorithm of the universal calculating program used for the calculation of the protonation constant of the 2,9-dimethylphenanthroline complex and for the subsequent calculations of the formation constants of the mixed-ligand complex with nucleotides can be reduced to the realization of the nonlinear least-square method adapted for the processing of spectral data.[2] The studied $CoL-H^+$ and $CoL-ATP-H^+$ systems are equilibrium mixtures of the initial components and possible compounds. When the spectrophotometric method is used, the measured property of the system, which reflects its composition, is the optical density *D* of the absorption of solutions at the given wavelength, the function of concentrations of all particles presenting in the system and their molar extinction coefficients (MEC). Under these conditions, it is necessary to calculate simultaneously spectral parameters and formation constants of compounds. Specifying original approximations of unknown values in the terms of the assumed equilibrium model and minimizing iteratively the sum of the squares of deviations of D_{exp} from D_{calc} over all experimental points with account of the totality of the mass balance equations over components and equations of the law of mass action, one can obtain the estimates of the formation constants for compounds, the values of unknown MEC, calculate the equilibrium concentrations of all particles in the solutions. The discrimination of the variants of the models proposed with the use of statistical criteria is the final stage of the calculation.

This program was used for studying of the complex formation process between nucleotides and phenan-

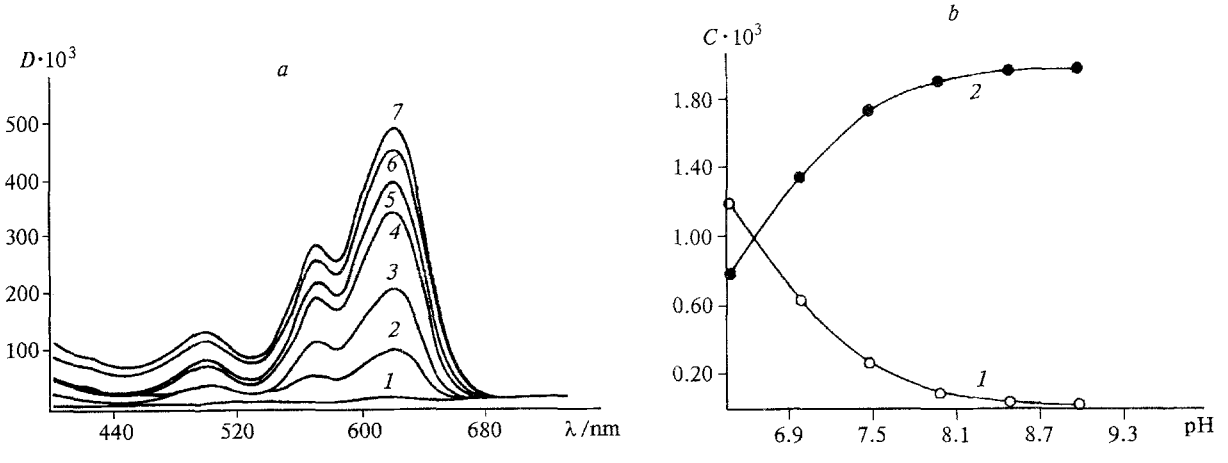

Fig. 3. pH effect on absorption spectra of the aqueous solution of the complex $[Co(2,9\text{-}Me_2phen)(H_2O)_2](NO_3)_2$.
a. pH-Titration of the complex $[Co(2,9\text{-}Me_2phen)(H_2O)_2](NO_3)_2$ in H_2O. Concentration of the complex is $5 \cdot 10^{-3}$ *M*; pH: *1*, 6.0; *2*, 6.5; *3*, 7.0; *4*, 7.5; *5*, 8.0; *6*, 8.5; *7*, 9.0.
b. Distribution curves of concentrations of the protonated (CoL, *1*) and deprotonated ($CoL(OH)^-$, *2*) forms of the complex depending on pH. Concentration of the complex is $5 \cdot 10^{-3}$ *M*.

throline complex CoL under the conditions close to those used in the studying of complexes in mitochondria. In order to improve solubility of the complex in an aqueous solution, the studies were performed in the presence of the 0.1% solution of Triton X-100, nonionic detergent, which does not change the absorption spectra of the solution, as it was shown by special experiments. In order to study the complex formation between CoL and ATP, a solution of the complex was added to the cuvette containing a phosphate buffer with 0.1% Triton X-100, the solution was stirred, the spectrum was registered, then the ATP solution was added, the mixture was stirred, and the spectrum was registered again. The pH values of the buffer solution varied from 7.0 to 9.0 and were thoroughly controlled in the course of the experiments.

As can be seen from Fig. 4, *a*, the addition of the growing amounts of ATP to the CoL complex decreases the absorption of the complex in the whole spectral range that allows one to suppose the interaction of the CoL complex with ATP to form a new compound. In order to confirm this assumption, the set of the spectral data obtained was processed by a personal computer by means of the program described above. The model including the following equilibria:

$$[CoL(OH)]^- + H^+ \rightleftharpoons CoL + H_2O$$

$$CoL + ATФ \rightleftharpoons CoL \cdot ATФ .$$

turned out to be the best model, which describes adequately the experimental data.

Figure 4, *b* shows the distribution curves of the concentrations of the complex forms in the system CoL—ATP—H^+ at varying pH and ATP concentration. The data obtained attest to the formation of the comparatively stable mixed complex CoL · ATP ($\log\beta = 4.45\pm0.21$) in the system, and the degree of formation of this complex is almost independent of pH (in the studied range from 7.0 to 9.0).

Thus, one can confirm that under the conditions of biochemical experiments with mitochondria, the efficient interaction is possible between ATP and coordinationally unsaturated complex CoL, which exists both in protonated and deprotonated forms.

We obtained the similar data for the complex formation between CoL and ADP ($\log\beta = 4.40\pm0.19$).

It is well known that coordination compounds of cobalt with macrocyclic and chelating ligands, in particular, with *o*-phenanthroline, are capable of reversible oxygen fixation.[3] In the CoL complex, the metal has a coordination number 6 and contains 4 easily eliminated coordinational sites. Our experiments showed that 1 mole of ATP was combined per 1 mole of the CoL complex. One may suppose that the ATP molecule occupies two coordination sites, and the coordination is

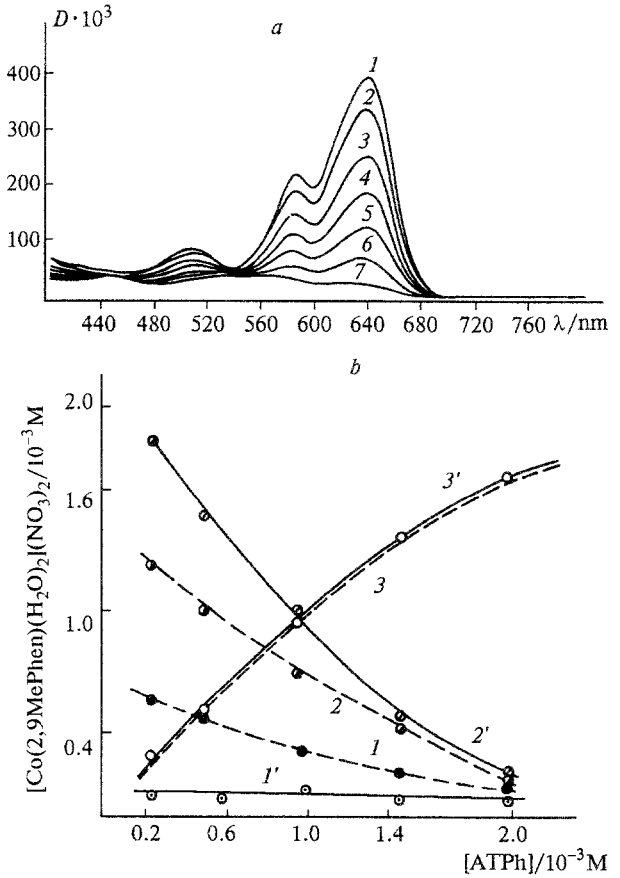

Fig. 4. Interaction of the complex [Co(2,9-Me_2phen)$(H_2O)_2$]$(NO_3)_2$ with ATP.
a. Titration of the solution of the complex [Co(2,9-Me_2phen)$(H_2O)_2$]$(NO_3)_2$ (CoL) with nucleotide (ATP) in the phosphate buffer: *1*, without ATP; *2—7*, in the presence of ATP, m*M*: *2*, 0.5; *3*, 0.75; *4*, 1.0; *5*, 1.5; *6*, 1.75; *7*, 2.0. Conditions of the reaction: KH_2PO_4 $5\cdot10^{-3}$ *M*; Triton X-100 0.1 %; pH 7.5, CoL $2\cdot10^{-3}$ *M*.
b. Distribution of different forms of complexes in the system CoL—ATP—H^+ depending on pH and ATP concentrations. *1*, *1'* — protonated form (CoL), *2*, *2'* — deprotonated form $[CoL(OH)]^-$, *3*, *3'* — mixed-ligand form (CoL · ATP). pH 7.0 (*1*, *2*, *3*) and 9.0 (*1'*, *2'*, *3'*).

performed *via* oxygen atoms of phosphate groups similarly to the ATP complexes with ammoniate cobalt complexes $[Co(NH_3)_4]^-$ · ATP, as it was shown in Ref. 14. It is of sense to assume that one more vacant coordination site could be occupied by the oxygen molecule, that would result in the formation of the mixed triple complex CoL · ATP · O_2.[9]

At first we compared the ability of the coordinationally unsaturated cobalt(II) complexes with *o*-phenanthroline and 2,9-dimethyl-*o*-phenanthroline to fix oxygen with the formation of superoxide radical anion $O_2^{\cdot -}$. For this purpose, a reduction reaction of nitroblue tetrazolium (NBT^{2+}) by superoxide into formazan in the presence of the cyanide ion has been used. The change in the solution color indicated to the existence of superoxide:

the reduction by the $O_2^{\cdot -}$ results in blue color of the initial solution of NBT^{2+}. The existence of superoxide in the triple cobalt complex with unsaturated phenanthroline and ATP was shown by this method previously.[10]

The spectra of the solutions containing cobalt complexes $[Co(2,9\text{-}Me_2phen)(H_2O)_2](NO_3)_2$ and $[Co(Phen)(H_2O)_2](NO_3)_2$ in the phosphate buffer (pH 7.8) in the presence of nitroblue tetrazolium and KCN are presented in Fig. 5, *a*. It can be seen that the formation of formazan, *i.e.*, reduction of NBT^{2+} under the action of O_2, was observed in the presence of $[Co(Phen)(H_2O)_2](NO_3)_2$ only (Fig. 5, *a*, curve *2*) and was not revealed in the presence of the complex $[Co(2,9\text{-}Me_2phen)(H_2O)_2](NO_3)_2$ (Fig. 5, *a*, curve *1*). It is likely that the existence of methyl substituents at positions 2 and 9 of the phenanthrolie ligand, which are in the direct vicinity of the chelate node, affects the ability of the complex to fix oxygen. The similar observations were made previously for the copper complexes with 2,9-dimethylphenanthroline.[15]

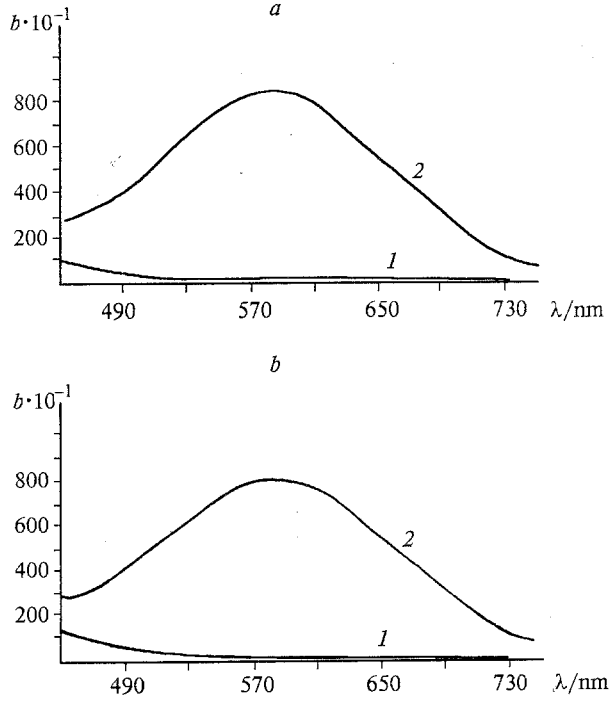

Fig. 5. Reduction of nitroblue tetrazolium (NBT^{2+}) by the superoxide ion in the presence of *o*-phenanthroline cobalt complexes.

a. Absorption spectra of formazan in the presence of the complexes $[Co(2,9\text{-}Me_2phen)(H_2O)_2](NO_3)_2$ (*1*) and $[Co(phen)(H_2O)_2](NO_3)_2$ (*2*). Conditions of the reaction: KH_2PO_4 $5 \cdot 10^{-2}$ *M* (pH 7.5); Triton X-100 0.025 %; CoL $0.25 \cdot 10^{-3}$ *M*; NBT^{2+} $0.25 \cdot 10^{-4}$ *M*; KCN $8 \cdot 10^{-3}$ *M*. The reaction was begun by the addition of KCN.

b. Absorption spectra of formazan in the presence of nucleotide-containing complexes.

1, $[Co(2,9\text{-}Me_2phen)H_2O](NO_3)_2 \cdot ATP$;

2, $[Co(phen)(H_2O)](NO_3)_2 \cdot ATP$.

Concentration of complexes is $0.25 \cdot 10^{-3}$ *M*. Conditions of the reactions see for Fig. 5, *a*.

We found that the introduction of a nucleotide molecule (ATP) into the composition of the complexes studied does not affect their ability to fix oxygen. As it is seen from Fig. 5, *b*, the cobalt complex with *o*-phenanthroline and ATP reduces nitroblue tetrazolium that indicates to the presence of the active particles $O_2^{\cdot -}$ in the coordination sphere of this mixed complex (curve *2*). The cobalt complex containing 2,9-dimethylphenanthroline and ATP does not reduce NBT^{2+} and, hence, does not contain oxygen in the coordination sphere (curve *1*).

These data allow one to conclude that the observed inhibition of the ATPase of mitochondria by the CoL complex is caused, first of all, by the inclusion of the nucleotide molecule into the coordination sphere of the complex. The complex CoL·ATP formed under the conditions of oxidative phosphorylation measuring in mitochondria, being an analog of the substrate of the ATPase reaction, provides the specific fixation of the complex in the active center of the enzyme and acts as an affine inhibitor.

The results obtained show that coordinationally unsaturated cobalt complexes with *o*-phenanthroline and its derivatives are promising for preparation of biologically active compounds, because allow one to include biologically significant molecules into the coordination sphere of cobalt, that provides a high specificity of the action of such complexes upon biosystems.

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