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A spectroscopic study of the reaction of the iron thiosulfate-nitrosyl complex with adenosine triphosphoric acid

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The reaction of the nitric oxide donor, *viz.*, iron tetranitrosyl complex bearing the thiosulfate ligand (TNIC), with adenosine triphosphoric acid (ATP) was studied. By spectrofluorometry and electronic microscopy, the formation of the reaction products of ATP both with TNIC and with $Na_2S_2O_3$ and $FeSO_4$ was shown and the complexation constants of ATP with these compounds were calculated.

Key words: adenosine triphosphoric acid, nitrosyl iron complexes, nitric oxide donors, fluorescence.

It is known that adenosine triphosphoric acid (ATP) is of vital importance in the energy and substance metabolism in organisms: the molecule of ATP contains two high-energy bonds and serves as a universal energy source for a great number of energetically expensive biochemical and physiological processes.[1] Such an important role of ATP in an organism determines the attention to this nucleotide of biologists and physicians. In recent years, the development of cellular and molecular biology favored deeper insight into the essence of myocardial trophic processes upon chronic cardiac insufficiency. These studies show that the mechanisms of cardiac dysfunction involve structural and functional remodeling not only of all compartments of the cardiovascular system (heart, arteries, and veins), but also of all tissue and cellular levels and elements (cardiomyocytes, smooth muscle cells, endothelium, extracellular matrix, membrane proteins, intracellular organelles, messenger systems *etc.*)[2]. In particular, the study of the regulation mechanisms of energy metabolism and stabilization of the ATP level in cells upon different forms of cardiovascular, lung, and blood diseases, as well as the study of the ageing and other processes is of current concern for modern biology and medicine.[3—7]

The interest in iron complexes is caused by the fact that many of them (*e.g.*, Fe^{III} complexes with nicotinamide) are drugs[8] or potential drugs.[9—11] In the present work, we studied the nitrosyl iron complex with sodium thiosulfate $Na_2[Fe_2(S_2O_3)_2(NO)_4] \cdot 4H_2O$ (TNIC). This complex is an efficient donor of nitric oxide and exhibits potential therapeutic activity.[10,11] For example, it has been shown *ex vivo* and *in vivo* on the models of the myocardial ischemic and reperfusion injury of the *Wistar* rats[10] that TNIC possesses a vasodilating effect, which is manifested in the efficient recovery of coronary flow and the decrease in the systolic blood pressure. This complex improves recovery, metabolism (in this case, the ATP level is 1.7-fold higher than in the control), and cardiac function after ischemia and decreases the size of myocardial infarction without arrythmogenic action, which can result in the death of experimental animals. The obtained data allow one to consider that this complex can be used as an original drug for the therapy of acute coronary syndrome. It is known[12] that the Fe^{III} complexes are able to form stable chemical bonds with ATP. All these facts cause fundamental interest in the behavior of TNIC towards the major macroergic compound, "key unit" of the energy metabolism, *viz.*, ATP, and in the study of intermediates produced.

The aim of the present work is to study the reaction of ATP and TNIC, which is promising nitric oxide donor, by spectrofluorometry.

Experimental

The commercially available ATP, $Na_2S_2O_3 \cdot 5H_2O$, and $FeSO_4 \cdot 7H_2O$ (99% purity, Sigma) were used as purchased.

The complex $Na_2[Fe_2(S_2O_3)_2(NO)_4] \cdot 4H_2O$ was synthesized according to the published procedure.[13]

All experiments were performed in a buffer with pH 6.8, for the preparation of which Tris • HCl (Sigma) was used.

Electronic absorption spectra were recorded on a Perkin—Elmer UV-VIS Spectrometer Lambda EZ 210 spectrophotometer. Absorption spectra at different concentration of ATP were recorded with compensation, the Tris • HCl (pH 6.8) buffer was used for compensation.

Spectrofluorometric measurements were performed on a Perkin—Elmer LS-55 spectrofluorometer. A known procedure[12] was

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used for the study of the reaction of ATP with the nitrosyl iron complex. The fluorescence excitation wavelength was $\lambda_{ex} = 290$ nm, 3—3.5 mL quartz cells were used, the optical path length was 1 cm, and the temperature was 20 °C. The fluorescence emission maximum was at $\lambda = 388$ nm. The range of working concentrations of ATP was determined experimentally. The linear variation in the fluorescence emission intensity is in the range of ATP concentrations from $2.0 \cdot 10^{-4}$ to $0.1 \cdot 10^{-4}$ mol L^{-1}.

The studied compounds $Na_2S_2O_3 \cdot 5H_2O$, $FeSO_4 \cdot 7H_2O$, and TNIC have no intrinsic fluorescence.

The effects of $Na_2S_2O_3$ and $FeSO_4$ on the change in the fluorescence of ATP were studied by continuous spectrofluorometric titration. Titration of a solution (3 mL) of ATP with a solution of the compound under study was performed in a measuring cell and the value of fluorescence emission was measured in the wavelength range of $\lambda = 380$—390 nm. A fluorescence spectrum was recorded after addition of each subsequent portion of a quenching agent. The time from addition of quencher to spectrum recording was no longer than 10—15 s. The time of recording the fluorescence value in the emission wavelength range of $\lambda = 380$—390 nm was also no longer than 10—15 s. The starting concentration of ATP was $0.959 \cdot 10^{-4}$ mol L^{-1}. The starting concentration of $Na_2S_2O_3$ was $0.887 \cdot 10^{-2}$ mol L^{-1}. The starting concentration of $FeSO_4$ was $0.953 \cdot 10^{-2}$ mol L^{-1}. A solution of quencher was added portionwise per 0.05 mL.

The measurement of the change in the fluorescence intensity of ATP under the action of TNIC was performed in each initial time point at different starting concentration of TNIC. Eight cells containing a solution of ATP (3 mL) with the concentration of $0.901 \cdot 10^{-4}$ mol L^{-1} were used in the experiments. Different volumes of a solution of TNIC with the starting concentration $1.007 \cdot 10^{-2}$ mol L^{-1} were added successively to each cell. After addition of TNIC, the fluorescence value was recorded at $\lambda = 380$—390 nm. Then, another portion of TNIC was added to the next cell, the fluorescence intensity was recorded *etc*. The time from the preparation of a working solution of TNIC to its addition to a solution of ATP did not exceed 1 min. The time between the measurements in different cells did not exceed 40—60 s.

Results and Discussion

In order to prove that ATP is a single fluorescent compound in the system and to prove the purity of working solutions of fluorescing agent, preliminary studies were performed. For this purpose, the absorption spectra and fluorescence excitation spectra of ATP were recorded (Fig. 1). The absorption spectrum of ATP has a maximum at $\lambda = 275$ nm and its fluorescence excitation spectrum has a maximum at $\lambda = 280$ nm. As these spectra are very close to each other, it is possible to claim that under the experimental conditions the change in the fluorescence intensity corresponds to that of fluorescence ATP, which is a single fluorescing compound present in a solution.

Figure 2 shows typical fluorescence spectra of ATP. As it can be seen, the position of the fluorescence maximum does not change upon decrease in the TNIC concentration in a solution. The decrease in the fluorescence emission intensity in this case is related to the decrease in the concentration of a luminous substance, *viz.*, ATP.

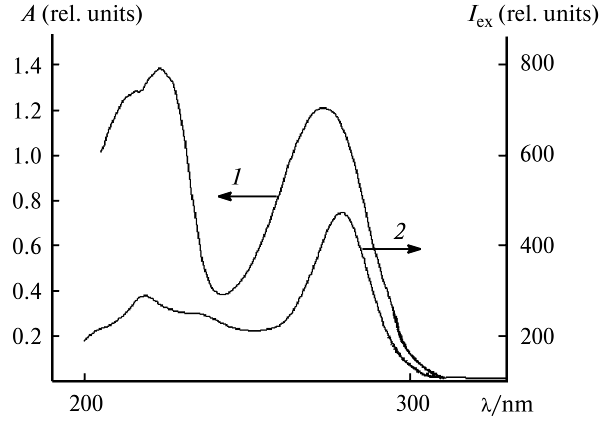


Fig. 1. The absorption (*1*) and fluorescence excitation (*2*) spectra of ATP ($c = 0.9014 \cdot 10^{-4}$ mol L^{-1}) in the Tris • HCl buffer (pH 6.8).

We studied the effects of TNIC, sodium thiosulfate ($Na_2S_2O_3$), and iron(II) sulfate ($FeSO_4$) (the starting compounds for the synthesis of the complex) on the fluorescence intensity of ATP. It was shown that all compounds under study decrease the fluorescence intensity of ATP (Fig. 3). It is obvious that in all cases under consideration, due to low working concentration of the compounds under study (~10^{-4} mol L^{-1}), the decrease in the fluorescence intensity of ATP is a result of the chemical reaction of the compound with ATP. This leads to a decrease in the concentration of free ATP molecules in the excited state. The change in the fluorescence by the other reasons (for example, *via* the mechanism described by the Stern—Volmer equation[14]) is unlikely as requires higher concentration of a quenching agent (1—0.1 mol L^{-1}).

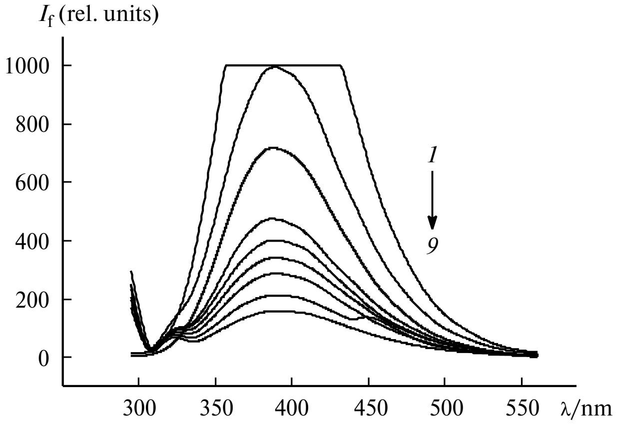


Fig. 2. The fluorescence spectra of ATP in the Tris • HCl buffer (pH 6.8) at the excitation wavelength $\lambda_{ex} = 290$ nm at the concentrations: $1.66 \cdot 10^{-2}$ (*1*), $8.33 \cdot 10^{-3}$ (*2*), $1.52 \cdot 10^{-4}$ (*3*), $7.61 \cdot 10^{-5}$ (*4*), $6.84 \cdot 10^{-5}$ (*5*), $6.08 \cdot 10^{-5}$ (*6*), $5.067 \cdot 10^{-5}$ (*7*), $3.55 \cdot 10^{-5}$ (*8*), and $2.53 \cdot 10^{-5}$ mol L^{-1} (*9*).

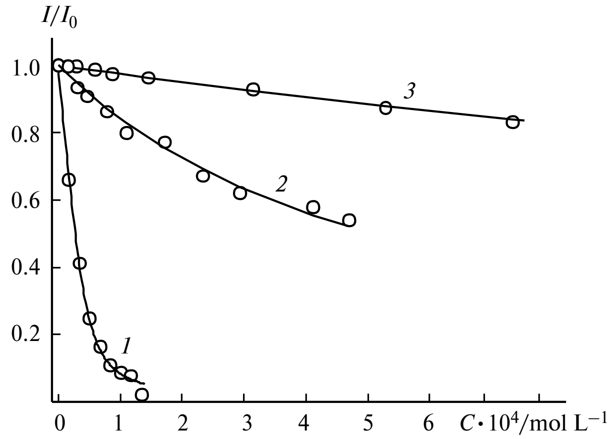


Fig. 3. Changes in the fluorescence intensity of ATP depending on the concentrations of TNIC (*1*), $FeSO_4$ (*2*), and $Na_2S_2O_3$ (*3*). The points are experimental data and the curves are calculation data. The experimental conditions are given in Experimental.

It follows from the experimental conditions that the reactions of the compounds under study with ATP proceeds in less than 10 s. A stable equilibrium is reached between the components of the system. It is seen from Fig. 3, curves *2* and *3*, that the starting compounds decrease the fluorescence intensity of ATP to a lesser extent than the complex TNIC. Based on the data obtained, one can assume that ATP reacts with TNIC and/or its dissociation products.

To confirm additionally the formation of new chemical compounds upon the reaction of ATP and TNIC, we performed a study of the components of the system by electronic absorption spectroscopy. The absorption maximum of ATP (Fig. 4, curve *1*) is observed at 275 nm. The absorption spectrum of TNIC has two well-defined separate maxima at 310 and 360 nm (Fig. 4, curve *2*).

The absorption spectrum of a mixture of ATP and TNIC (see Fig. 4, curve *3*), like the spectrum of pure TNIC, displays maxima at 310 and 360 nm. However, in contrast to the spectrum of ATP, the spectrum of the mixture exhibits a bathochromic shift of the maximum at 275 nm, which is typical of ATP, by about 20 nm. Thus, according to the changes in the electronic spectra, one can assume that TNIC reacts with ATP.

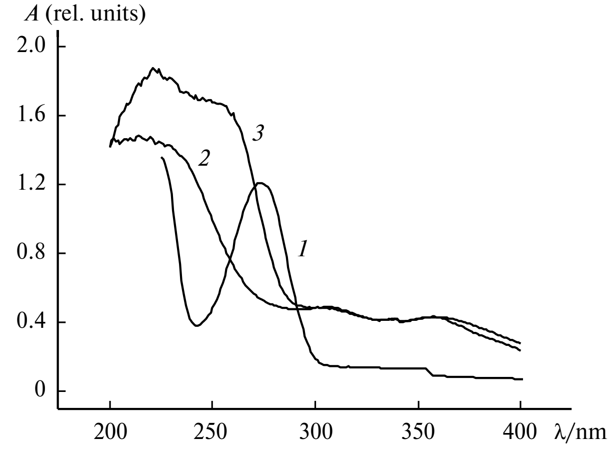
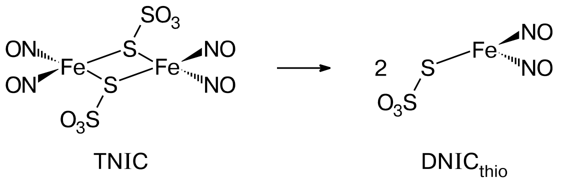


Fig. 4. The absorption spectra of the complex of ATP (*1*), TNIC (*2*), and a mixture of ATP and TNIC (*3*).

The analysis of the obtained experimental data was based on the available research data[11] indicating that the binuclear complex TNIC undergoes primary breakage in protic media to form mononuclear dinitrosyl iron complexes ($DNIC_{thio}$). Then, $DNIC_{thio}$ decomposes to mononitrosyl complex and other hydrolysis products to evolve nitric oxide into a solution. The structures of these intermediates were confirmed by mass spectrometry[15] (Scheme 1).

Scheme 1



The measurement of the decomplexation rate of $DNIC_{thio}$ showed that its lifetime is significantly longer than the reaction time for interaction of ATP with $DNIC_{thio}$ found by spectrofluorometry. Consequently, the reaction between ATP and $DNIC_{thio}$ can be expressed as the kinetic equation:

$$\mathrm{ATP} + \mathrm{DNIC_{thio}} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{P},$$

where P is a reaction product, *viz.*, the complex [ATP-$DNIC_{thio}$], k_1 and k_{-1} are the rate constants of forward and reverse reactions, respectively, and $K = k_1/k_{-1}$ is the equilibrium constant or complexation constant of the complex [ATP-$DNIC_{thio}$].

The data from spectrofluorometric titration allowed calculation the complexation constants (K) of TNIC, $Na_2S_2O_3$, and $FeSO_4$ with ATP, *i.e.*, determination of the stability of their complexes with ATP.

Compound	K/mol L^{-1}
$Na_2S_2O_3$	$(2.69\pm0.04) \cdot 10^2$
$FeSO_4$	$(2.18\pm0.09) \cdot 10^3$
$Na_2[Fe_2(S_2O_3)_2(NO)_4]$ (TNIC)	$(9.20\pm0.70) \cdot 10^4$

The highest K value was found for TNIC. The stability of its complex with ATP is by one order of magnitude higher than that for ferric sulfate and by four orders of magnitude higher than that for sodium thiosulfate. From the K values obtained, the theoretical curves given in Fig. 2 were calculated. As it seen, the calculated constant is in good agreement with the experimental results.

Obviously, the resulted difference in the *K* values reflects the chemical structures of the compounds. In the molecule of sodium thiosulfate, the sulfur and sodium atoms have no free valences for additional bonding. The dissociation of the S—S bond in a solution results in the formation of Na_2S and $(SO_3)^-$. Probably, the former can give ionic compound with the hydrogen atom of the terminal NH_2 group of adenine. In a solution, $FeSO_4$ dissociates also. Certainly, the degree of its dissociation is higher than that of $Na_2S_2O_3$. The ability of iron to form coordination bonds is manifested in the reaction of ATP with TNIC. The three-order-of-magnitude difference in the *K* values for $FeSO_4$ and TNIC suggests the formation of a coordination compound. Theoretically, the Fe^{+2} ion can react with the phosphoric acid residues. However, as stated above, the dissociation rate of TNIC in a solution is lower than the reaction rate of ATP with TNIC. This can mean that the ATP molecule reacts with the producing intermediate $DNIC_{thio}$.

One can assume that in a solution, addition of TNIC to the adenine part of the molecule responsible for the fluorescent properties of ATP occurs. The analogous reaction with the adenine part of the nucleotide involving the electrons of the internal coordination sphere of iron atom has been shown earlier[12] by the example of the complex $[FeL_2]$ (L is 3,6-dichloropicolinic acid). However, the exact determination of the compound obtained and the possible reaction mechanism of ATP with TNIC requires additional studies.

Thus, in the present work, we established that ATP reacts with thiosulfate nitrosyl iron complex, which is a donor of nitric oxide. The proposed kinetic model allows calculation of the complexation constants.

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