MATHEMATICAL MODELING OF BIOCHEMICAL PROCESS

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KINETIC REGULARITIES AND A POSSIBLE MECHANISM OF ATP NON-ENZYMATIC HYDROLYSIS INDUCED BY CALIX[4]ARENE C-107

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The kinetic model of calix[4]arene-induced ATP hydrolysis was elaborated. It is assumed that calix[4]-arene C-107 molecules form a complex with nucleoside triphosphate, ensuring the release of inorganic phosphate P_i, and then switch into an inactive state. Inactive calix[4]arene molecules are no longer able to form a complex with ATP and, accordingly, to provide hydrolysis of nucleoside triphosphate. In the author's experimental studies, it was possible to explain the kinetic properties of the reaction, namely: the insignificant output of the reaction end product P_i; the quantitative regularities of the plateau (time-wise) accumulation of the reaction product when the concentration of calix[4]arene C-107 or ATP changes; the reciprocal dependence of the ATP half conversion on its concentration; the correspondence of the initial reaction rate dependence on the calix[4]arene and ATP concentration to the Michaelis-Menten equation. The final decision regarding the molecular mechanism of calix[4]arene-induced ATP hydrolysis requires further experimental and theoretical studies.

K e y w o r d s: hydrolysis of ATP, calixarenes, empirical kinetic analysis.

It is well known that adenosine-5′-triphosphate (ATP) is the main intracellular energetic "currency"; this substance is the primary source of energy and its transmitter in the living organisms. For instance, the energy released during the enzymatic hydrolysis of ATP, conducted by vector electro-enzymes – ATP-hydrolase cation-transporting systems (electrogenic sodium and calcium pumps) and "mechanochemical" ATP-hydrolases, is used to create transmembrane cation gradients (Ca^{2+}, Na^+, K^+) and ensure the contraction of muscles and functional activity of motor cells. In our previous experiments, conducted on membrane structures of smooth muscle (SM) cells, we demonstrated that some calix[4]arenes can selectively and effectively inhibit the enzymatic activity of cation-transporting ATP-hydrolases, localized in the plasma membrane (PM) [1-3].

For instance, calix[4]arenes C-97 (5-bis(dihydroxyphosphoryl)methyl-26,28-dihydroxy-25,27-dipropoxycalix[4]arene), C-99 (5,17-bis(dihydroxyphosphonylmethylol)-26,28-dihydroxy-25,27-dipropoxycalix[4]arene), and C-107 (5,17-di(phosphono-2-pyridylmethyl)amino-11,23-di-tert-butyl-26,28-dihydroxy-25,27-dipropoxy-calix[4]arene) show high affinity (the inhibition coefficient $I_{0.5}$ < 100 nM) in inhibiting the activity of Na^+,K^+-ATPase (sodium pump) of the plasma membrane – Mg^{2+},Na^+,K^+-

dependent Ca^{2+}-independent electro-enzyme from the group of transport adenosine triphosphatases, which performs active electrogenic antiport transfer of monovalent cations in the mode of $3Na^+$:$2K^+$ exchange, creating the sodium and potassium transmembrane gradients, directed from the extracellular medium into the cell and outside from the cell, respectively. However, the mentioned calix[4]arenes demonstrated the effect of selective inhibition specifically on Na^+,K^+-ATPase. They did not impact the activity of other ATP-hydrolases, localized in this subcellular structure – thapsigargin-resistant electrogenic Ca^{2+},Mg^{2+}-dependent ("ATP-dependent calcium pump"), Ca^{2+}-independent Mg^{2+}-dependent ("basal"), and Mg^{2+}-independent Ca^{2+}-dependent one [4-7].

On the other hand, calix[4]arenes C-90, C-956, and C-1087 selectively (regarding other ATP-hydrolases – Na^+,K^+-dependent ("ATP-dependent sodium pump"), Ca^{2+}-independent Mg^{2+}-dependent ("basal"), Mg^{2+}-independent Ca^{2+}-dependent), and rather affinely ($I_{0.5}$ ~ 10–50 μM)) inhibited the activity of thapsigargin-resistant electrogenic Mg^{2+}, ATP-dependent calcium pump of the plasma membrane, induced the elevation in the concentration of ionized calcium in the myoplasm and the level of Ca^{2+}-dependent oxytocin-induced contractions of myometrium [1, 8]. Thus, taking into consideration the fact that usually, calix[4]arenes are substances of low toxicity [9, 10], calix[4]arenes C-90, C-956, and C-1087 can potentially be viewed as possible factors of enhancing the contractility of the uterus if the stimulation of this smooth muscle organ with oxytocin during delivery is insufficient, and thus, there is a risk of cesarean section to save the fetus.

However, in the experiments described above, we noted an interesting phenomenon. We determined [12, 13] that some calix[4]arenes can form complexes with ATP and hydrolyze the nucleoside triphosphate in the absence of subcellular membrane structures in the incubation medium (the concentration of calix[4]arenes – 100 μM, the process of nucleoside triphosphate hydrolysis was tested by the release of one product of the mentioned reaction – inorganic phosphate P_i). We found that among the calix[4]arenes in our research, the most effective stimulation of non-enzymatic hydrolysis of ATP was demonstrated by calix[4]arene C-107 (5,17-di(phosphono-2-pyridylmethyl)amino-11,23-di-tert-butyl-26,28-dihydroxy-25,27-dipropoxy-calix[4]arene), the level of nucleoside triphosphate hydrolysis was 17.3 ± 1.3 nmol P_i in 20 min. The incubation of calix[4]arene C-160 (5,11-di(phosphono-2-pyridylmethyl)amino-17,23-di-tert-butyl-26,27-dihydroxy-25,28-dipropoxy-calix[4]arene) was twice less effective in stimulating the ATP hydrolysis, and calix[4]arene C-150 (upper rim unsubstituted 26,28-dihydroxy-25,27-dipropoxycalix[4]arene) was practically unable to hydrolyze ATP. In the independent study, other authors used the methods of laser photolysis and pulse radiolysis to prove the availability of the complex between calix[4]arene (using calix[4]arene-5,11,17,23-tetrasulfonate as an example) and ATP [14]. Also, recently, the fact of the complex formation between calix[4]arene C-107 and ATP was confirmed in the experiments, conducted using the fluorescence methods [15].

Some properties of the calix[4]arene-induced non-enzymatic hydrolysis of ATP were described by us in the previous papers [2, 13]. For instance, the value of the activation energy E_a of the calixarene-induced hydrolysis of ATP was 50.7 + 8.9 kJ/mol [13]. However, the mentioned reaction had noteworthy specificity. It was found that after the reaction completion, the time-wise maximal ("plateau") amount pmax of inorganic phosphate P_i, released due to calixarene-induced hydrolysis of ATP was considerably smaller than the initial amount of ATP in the incubation medium. Therefore, the termination (practically since the 45th minute of incubation) of the hydrolysis reaction, induced by calix[4]arene C-107, cannot be conditioned by possible complete exhaustion of ATP during the reaction; it is regulated by some other factor.

To elaborate the ideas on a possible mechanism and interpretation of the properties of calix[4]arene-induced hydrolysis of ATP (using calix[4]arene C-107), we set the task to develop the kinetic model of this reaction, which would explain the specificity of its course.

Materials and Methods

The study of the formation of a calix[4]arene C-107–ATP complex. A detailed description of the synthesis (as a mixture of stereoisomers) and the spectral characteristics of calix[4]arene C-107 (Fig. 1) using the methods of nuclear magnetic resonance and infrared spectroscopy [6].

The method based on the application of the reversed-phase high-performance liquid chromatography was used [12] to study the formation of a calix[4]arene C-107–ATP complex. This method

enables the work with low concentrations of the investigated substances in polar solvents and their mixtures. To enhance the reliability of the study results, two types of chromatographic columns of different polarity (Zorbax CN and LiChrosorb RP 18), were applied according to chemical properties of calix[4]arene C-107 and ATP, as well as available scientific literature data [2, 16] on the analysis of similar substances. The complex formation was studied in the solution of acetonitrile and water (47/53 in the volume) used as a mobile phase. During the chromatographic analysis, the introduction of ATP to the composition of the mobile phase of calix[4]arene C-107 resulted in the formation of a "Host-Guest" complex with the 1:1 stoichiometry and caused the decrease in the values of the main chromatographic characteristics of ATP: retention time t_R and capacity coefficient. A detailed procedure for the corresponding calculations was presented in the article [17]. It was found that the values of dissociation constants *K*, calculated by this method, were 231 and 197 μM for LiChrosorb RP 18 and Zorbax CN columns, respectively. In our further kinetic evaluations, we accepted K = 215 μM as the average value.

As stated above, other authors used the methods of laser photolysis, pulse radiolysis and spectrofluorimetry to prove the formation of complexes between calix[4]arenes and ATP [14, 15].

The study of the non-enzymatic hydrolysis of ATP induced by calix[4]arene C-107. The reaction of non-enzymatic hydrolysis of ATP, stimulated by calix[4]arene C-107, was studied as previously described [2]. The incubation medium (volume – 0.4 ml) contained (mM): 1 ATP, 3 $MgCl_2$, 50 NaCl, 100 KCl, 1 EDTA, 20 Hepes-tris-buffer (pH 7.4, 37°C). Usually, the concentration of calix[4]arene in the incubation medium was 100 μM, and the incubation lasted for 20 min if other conditions were not stated. The reaction was initiated by the introduction of 40 μl calixarene solution to the incubation medium and terminated by the addition of 1 ml of the "stop"-solution to the incubation medium (pH 4.3 at 8°C). The "stop"-solution consisted of 1.5 M acid sodium acetate, 3.7% formaldehyde, 14% ethanol, and 5% TCA. The experiments used the initial concentrated solutions of calix[4]arene C-107 (4 mM) in DMSO, diluted with distilled water up to the required concentration of calixarene.

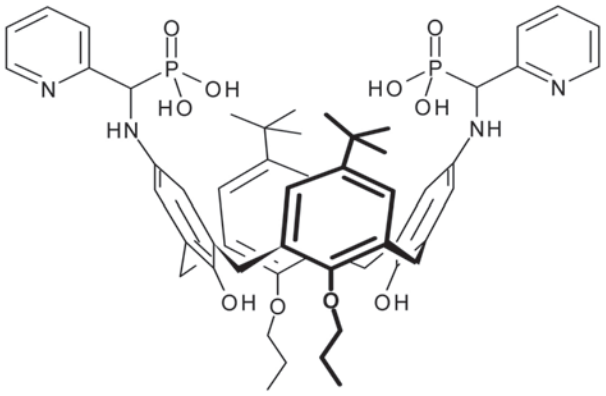

Fig. 1. The structural formula of calix[4]arene C-107 (5,17-di(phosphono-2-pyridylmethyl)amino-11,23-di-tert-butyl-26,28-dihydroxy-25,27-dipropoxycalix[4]arene)

Three controls for the content of inorganic phosphate P_i in the incubation medium were used in these experiments: 1) for the content of P_i, which was added to the incubation medium with the reagents (but on condition of the absence of ATP and calix[4]arene therein); 2) for the release of P_i due to spontaneous hydrolysis of ATP (in the absence of calix[4]arene) during the incubation; 3) for the possible increase in the content of P_i in the incubation medium as a result of the addition of calix[4]arene (in the absence of ATP). Thus, the ATP hydrolysis, stimulated by calix[4]arene C-107, was evaluated by the content of P_i, formed in the incubation medium in the presence of calix[4]arene, considering the correction for the release of phosphate under the spontaneous hydrolysis of ATP and the content of endogenous P_i in the incubation medium components. Usually, the amount of P_i, tested on the 20th minute of the incubation as a result of purely calix[4]arene-induced hydrolysis of ATP (17–19 nmol P_i in 20 min of incubation), exceeded the amount of P_i as a total of all three controls on average 2.5–3 times.

The amount of the reaction product P_i was determined by the method of W. Rathbun and V. Betluch [18].

The kinetic analysis of the non-enzymatic hydrolysis of ATP, induced by calix[4]arene C-107). The kinetic analysis of the ATP hydrolysis mechanism, induced by calix[4]arene C-107 was conducted in the permanent mode, using the standard principles of chemical kinetics.

Results and Discussion

The assumptions about the possible mechanism of the non-enzymatic ATP hydrolysis, ensured by calix[4]arene C-107. Previously we determined the fol-

lowing specificities of the ATP hydrolysis reaction induced by calix[4]arene C-107 [12, 13].

1. As stated above, the reversed-phase high-performance liquid chromatography was used to prove that the ATP molecule was capable of forming a complex with the molecule of calix[4]arene C-107 in the stoichiometric ratio of 1:1, the value of the dissociation constant K = 197–231 μM (as stated above, we accepted the value of K – 215 μM in our further work). Using HyperChem 7.01 program, the method of molecular mechanics ("the force field MM+"), and the semiempirical method ("CNDO field"), we managed to identify the force interactions, stabilizing the mentioned complex. There are grounds for the assumption that intermolecular hydrogen bonds of different types are formed between calix[4]arene C-107 and ATP. For instance, the protonated secondary amine group of one of the amino-(2-pyridyl) methylphosphonate substituents of calix[4]arene-107 may interact with γ- and β-phosphoryl residues of the ATP molecule. At the same time, the ammonium part of the second substituent may interact with the α-phosphoryl moiety and deoxyribose hydroxyl group of nucleoside triphosphate molecule. Finally, OH-group of one of the monoanionic phosphonate fragment may bind the γ-phosphoryl residue of the ATP molecule; at the same time, OH-group of the second phosphonate fragment of calix[4]arene C-107 may bind the β- or α-phosphoryl residue of the ATP molecule. It could not be ruled out that along with hydrogen bonds, some other interactions can also be responsible for stabilization of the C-107–ATP complex, such as π-π, OH–π, NH_2^+–π [2, 12].

2. It was found that the time-wise maximal ("plateau" on the 45th minute of the incubation) amount of inorganic phosphate p_{max} (~ 23 nmol), released due to the calixarene-induced hydrolysis of ATP, was much lower than the initial amount of r_0 ATP in the incubation medium (the initial concentration $[ATP]_0 = s_0$ = 1 mM, the volume of the incubation medium = 0.4 ml). Thus, the average value in the correlation $p_{max}/r_0 \approx 23$ nmol P_i/400 nmol ATP $\approx 6 \cdot 10^{-2} << 1$, i.e. the amount of P_i, released in the course of calixarene-dependent reaction of ATP hydrolysis, did not exceed 10% of the initial ATP amount in the incubation medium. Therefore, the spontaneous termination (practically since the 45th minute of incubation) of the ATP hydrolysis reaction, induced by calix[4]arene C-107, cannot be conditioned by possible complete exhaustion of ATP during the reaction; it is limited by some other factor. On the other hand, quite obviously, it is impossible to explain the highly efficient inhibiting effect ($I_{0.5}$ = 54 ± 6 nM) of the mentioned substance on the activity of Na^+,K^+-ATPase of the plasma membrane by the stimulation of the non-enzymatic hydrolysis of ATP under the effect of calix[4]arene C-107 [4, 6, 19]. Firstly, if the reason for the inhibiting effect of calix[4]arene C-107 on the mentioned enzyme lay in the trivial binding of ATP by this calix[4]arene (a kind of "release" of the substrate – ATP in ATP-hydrolase reaction), then the inhibiting effect of the enzymatic hydrolysis of ATP would be observed for all the other ATP-hydrolases of the plasma membrane – Ca^{2+},Mg^{2+}-dependent, Ca^{2+}-independent Mg^{2+}-dependent ("basal"), Mg^{2+}-independent Ca^{2+}-dependent. However, we observed evident selectivity in the inhibiting effect of calix[4]arene C-107 specifically on Na^+,K^+-ATPase; the activity of other ATP-hydrolases was resistant to the effect of this calix[4]arene [6]. Secondly, if one assumes that the ATP binding with calix[4]arene C-107 lays the grounds for the inhibiting effect on the activity of Na^+,K^+-ATPase, then, considering that in the incubation medium ATP remains in the concentration not lower than 90% of the initial concentration of nucleoside triphosphate s_0 (see above), the interpretation of this spontaneous inhibiting effect, coming from the idea of the formation of "calix[4]arene-ATP" complex, is obviously not reasonable.

3. The period of ATP half conversion $\tau_{0.5}$ under the stable concentration of calix[4]arene C-107 decreases in case of a higher concentration of ATP.

4. The velocity of the calixarene-induced hydrolysis of ATP elevated both after the increase in the concentration of calix[4]arene C-107 (under the stable concentration of ATP), and after the rise in the ATP concentration (under the stable concentration of calix[4]arene C-107).

5. It was found that the introduction of Mg^{2+} or Ca^{2+} ions into the incubation medium (1 mM concentration of the relevant salts) had practically no effect on the ATP hydrolysis reaction induced by calix[4]arene C-107 (pH = 7.4) [2, 13]. This fact may demonstrate that the calix[4]arene is capable of successful hydrolysis of ATP, which is either in a free state (ATP^{4-}) or in the form of chelate complexes ($MgATP^{2-}$, $CaATP^{2-}$). It is noteworthy that in case of enzymatic hydrolysis of ATP, ensured by transporting Mg^{2+}-dependent ATP-hydrolases, the real substrate of the reaction is specifically the chelate complex $MgATP^{2-}$ [20, 21].

Thus, taking into account these facts and statements:

- the formation of "calix[4]arene-C-107–ATP" complex (stoichiometry – 1:1);

- the termination (since the 45th minute of incubation) of the reaction of non-enzymatic hydrolysis of ATP, induced by calix[4]arene C-107, cannot be explained by possible complete exhaustion of ATP in the course of the reaction (since the maximal amount of inorganic phosphate p_{max}, released during the calixarene-dependent reaction of ATP hydrolysis, is much lower than the initial amount of ATP r_0);

- in the reaction sphere, the initial concentration of ATP s_0 (1–5 mM) is considerably higher than the initial concentration of calix[4]arene q_0 (20–100 μM): $s_0/q_0 \approx 10^2 >> 1$,

we had the following assumption: during the calixarene-induced non-enzymatic hydrolysis of ATP, the molecules of calix[4]arene C-107, which formed a complex with nucleoside triphosphate and ensured the release of inorganic phosphate P_i, switch into non-reactive states; being in these states, these "inactive" molecules of calix[4]arene C-107 cannot form a complex with ATP anymore and thus ensure the hydrolysis of nucleoside triphosphate. In other words, this entails the reaction of calixarene-induced hydrolysis of ATP, during which calix[4]arene C-107 switches to an inactive state, due to which the calixarene molecule loses its capability of ensuring the hydrolysis of nucleoside triphosphate, and thus the reaction terminated.

In the context of this idea in the simplest case, it can be accepted that the abovementioned considerations are in accordance with the following mechanism (1) of non-enzymatic calixarene-induced hydrolysis of ATP

$$\begin{array}{ccccc} & & k_1 & k & \\ Q + S & \rightleftharpoons & QS & \rightarrow Q' + P, \\ & & k_{-1}\ \downarrow k' & & \\ & & (QS)' & & \end{array} \qquad (1)$$

where Q – calix[4]arene C-107; S – cumulatively ATP and $MgATP^{2-}$ (because, as stated above, the availability of Mg^{2+} in the incubation medium has practically no effect on the reaction of calix[4]arene-induced hydrolysis of ATP); QS – the intermediate "calix[4]arene C-107–ATP" complex; P – a product of ATP-hydrolase reaction (inorganic phosphate P_i), the accumulation of which was used to trace its kinetics; Q′ – calix[4]arene C-107 in the inactive state, incapable of complex formation with ATP and hydrolysis of nucleoside triphosphate; (QS)′ – calix[4]arene C-107 in the complex with ATP, but also in the inactive state, incapable of hydrolysis of nucleoside triphosphate; k_1 and k_{-1} – the rate constants for the stages of association (the constant of the second order) and dissociation (the constant of the first order) respectively at the level of the formation of the intermediate complex QS (the constant of dissociation of this complex $K = k_{-1}/k_1$); k and k' – the rate constants of the first order for the relevant stages, resulting in inactive calixarene.

The kinetic interpretation of the possible mechanism of the ATP hydrolysis reaction ensured by calix[4]arene C-107. Fig. 2, panel *A* presents a typical experimental kinetic chart for the reaction of the non-enzymatic calixarene-induced hydrolysis of ATP. A principal question arises: can the postulated mechanism (1) actually provide a reliable description of the kinetics of this reaction? Panel *A* – a kinetic curve for the reaction of calixarene-induced hydrolysis of ATP. The concentration of C-107 and ATP were 100 μM and 1 mM, respectively, (the temperature 37°C, pH 7.4). Panel *B* – linearization (r^2 = 0.97–0.99), regarding the equation (13), a kinetic curve, presented in panel *A*. The plateau value of the accumulation of the reaction product p_{max} was accepted as 23 nmol (see panel *A*).

Let us conduct the kinetic analysis of the mechanism in the permanent mode.

It can be assumed that q_0, q and q' – a total concentration of calix[4]arene C-107 and its concentrations in the states QS and (QS)′ respectively, s_0 and s – a total concentration of ATP and the concentration of nucleoside triphosphate in free state (not in the complex with calix[4]arene), respectively, z and z' – the concentrations of complexes QS and (QS)' respectively, p – the concentration of the reaction product (inorganic phosphate P_i). Then, according to the principle of preserving the total concentration of the substance in the reaction sphere, we will have:

for the concentration of calix[4]arene C-107 –

$$q_0 = q + z + z' + q', \qquad (2)$$

for the concentration of ATP –

$$s_0 = s + p + z + z'. \qquad (3)$$

However, taking into account that, firstly, the total concentration of calix[4]arene C-107 q_0 in the incubation medium is considerably lower than the total concentration of ATP s_0 (q_0/s_0 = 100 μM/1 mM = 0.1 << 1), and, secondly, in the reaction sphere (volume of 0.4 ml) after its completion, there is ac-

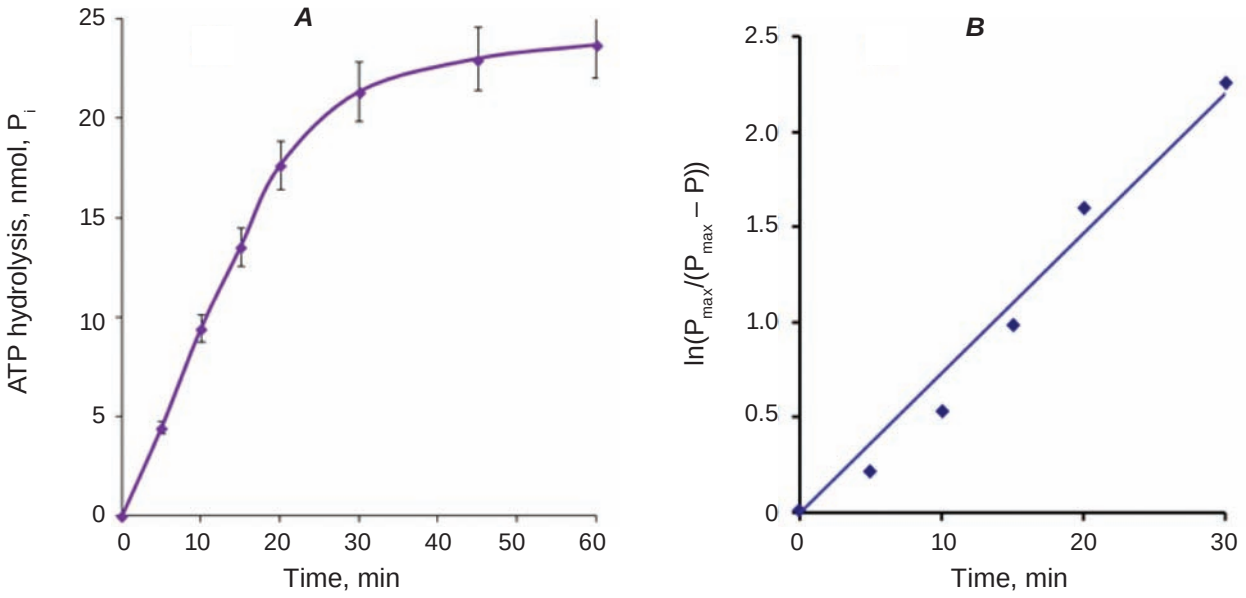

Fig. 2. To the kinetic analysis of the possible mechanism (1) of the ATP hydrolysis reaction induced by calix[4]arene C-107 (M ± m; n = 5)

cumulation of the product P p_{max}, there is very little relatively total amount r0 of nucleoside triphosphate ($p_{max}/r_0 \approx 23$ nmol P_i/400 nmol ATP = $6 \cdot 10^{-2} << 1$), we can transform the equation (3) in some approximation as follows:

$$s_0 \approx s. \quad (4)$$

Taking into account the constant of the dissociation of the intermediate complex QS and considering (4), the concentration of this complex is determined as:

$$K = k_{-1}/k_1 = qs/z = qs_0/z. \quad (5)$$

According to the scheme (1) we get the equations: $(dz'/dt)/(dq'/dt) = k'/k$, i.e.

$$z' = (k'/k)q', \quad (6)$$

and $dq'/dt = dp/dt$, i.e.

$$q' = p. \quad (7)$$

According to the scheme (1) for the instant velocity of the reaction of calixarene-induced hydrolysis of ATP $v = dp/dt$ can be defined as follows:

$$v = dp/dt = kz. \quad (8)$$

For the permanent mode (regarding the concentration of QS complex), we get, according to the method of Bodenstein and the equation (4):

$$(dz/dt)_{perm} = k_1 qs_0 - (k' + k + k_{-1})z = 0. \quad (9)$$

Considering the correlations (2) and (5) – (9) for the instant velocity of the reaction of calixarene-induced hydrolysis of ATP, we can get the following equation:

$$dp/dt = k[q_0 - (\gamma+1)p]s_0/\{[(k' + k)/k_1] + K + s_0\}, \quad (10)$$

where $\gamma = k'/k$ – a non-dimensional coefficient that characterizes the ratio between two rate constants of the first order at the level of the corresponding stages of transformation of calix[4]arene into the non-reactive ("inactive") states (see scheme (1)).

The integration of equation (10) results in the equation for explicit dependence (11) of the accumulation of the reaction product p in time (conditions: in case of time $t = 0$, the accumulation of the reaction product $p = 0$, in case of any current value of time t we get the accumulation of the product p):

$$p/p_{max} = 1 - e^{-k(\gamma+1)s0t/\{[(k' + k)/k1] + K + s0\}}, \quad (11)$$

where the plateau (time-wise) value of the accumulation of the reaction product pmax is determined by the equation:

$$p_{max} = q_0/(\gamma + 1). \quad (12)$$

The equation (11) in the linearized form looks as follows:

$$\ln[p_{max}/(p_{max}-p)] = k(\gamma+1)s_0 t/\{[(k'+k)/k_1]+K+s_0\}. \quad (13)$$

As seen in Fig. 2, panel *B*, the initial kinetic chart, presented in Fig. 2, panel *A*, is well ($r^2 = 0.97 – 0.99$) linearized according to the correlation (13), in semi-logarithmic coordinates $\ln[p_{max}/(p_{max}-p)]; t$. This result demonstrates directly that equation (11), which corresponds to the postulated reaction mechanism (1), is a correct description

for the kinetics of the ATP hydrolysis reaction which is induced by calix[4]arene C-107 (Fig. 2, panel *A*) and thus the mechanism of this reaction, reflected at the level of the suggested scheme (1), can actually be real.

Regarding the maximal (time-wise) value of p_{max} for the released reaction product – inorganic phosphate P_i in case of the ATP hydrolysis process, ensured by calix[4]arene C-107. We found out that under the stable concentration of ATP (1 mM), the increase in the concentration of calix[4]arene C-107 (20–100 μM) resulted in the rise in the maximal ("plateau" time-wise, on the 45th minute of the incubation) amount p_{max} of the released reaction product – inorganic phosphate P_i. On the contrary, under the stable concentration of calix[4]arene C-107 (100 μM), the increase in the concentration of ATP (1–5 mM) did not result in the change in the maximal ("plateau" time-wise) level pmax of the released inorganic phosphate P_i (Fig. 3).

According to the results of our kinetic analysis, these facts find their actual explanation within the abovedescribed mechanism of the calixarene-induced hydrolysis of ATP. Using equation (12), we see that the plateau value of the amount of the accumulated reaction product pmax depends on the correlation $\gamma = k'/k$ (see scheme (1)), and it is determined solely by the magnitude of the total concentration of calix[4]arene C-107 q_0 (the higher q_0 – the higher p_{max}), but it does not depend on the total concentration of ATP s_0, which is completely in agreement with the experimental data (Fig. 3, [13]).

Regarding the period of half conversion $\tau_{0.5}$ in case of the ATP hydrolysis reaction, ensured by calix[4]arene C-107. We will get the equation for the period of half conversion of ATP $\tau_{0.5}$ in case of mechanism (1). Using the equation (11) for the time moment $\tau_{0.5}$, we see that $p = p_{max}/2$, so

$$\tau_{0.5} = [\ln 2/k(\gamma+1)] + [\ln 2/k(\gamma+1)]\{[(k'+k)/k_1]+K\}/s_0. \quad (14)$$

Therefore, according to the correlation (14), the results of the kinetic analysis of the mechanism (1) envisage that with the increase in the concentration of ATP s_0 the period of half conversion of nucleoside triphosphate $\tau_{0.5}$ under the effect of calix[4]arene C-107 should decrease. We actually observed this phenomenon in the experiment (Fig. 4 and [13]).

The evaluation of the coefficient values and individual constants of the velocity k and k' in case of the ATP hydrolysis reaction ensured by calix[4]arene

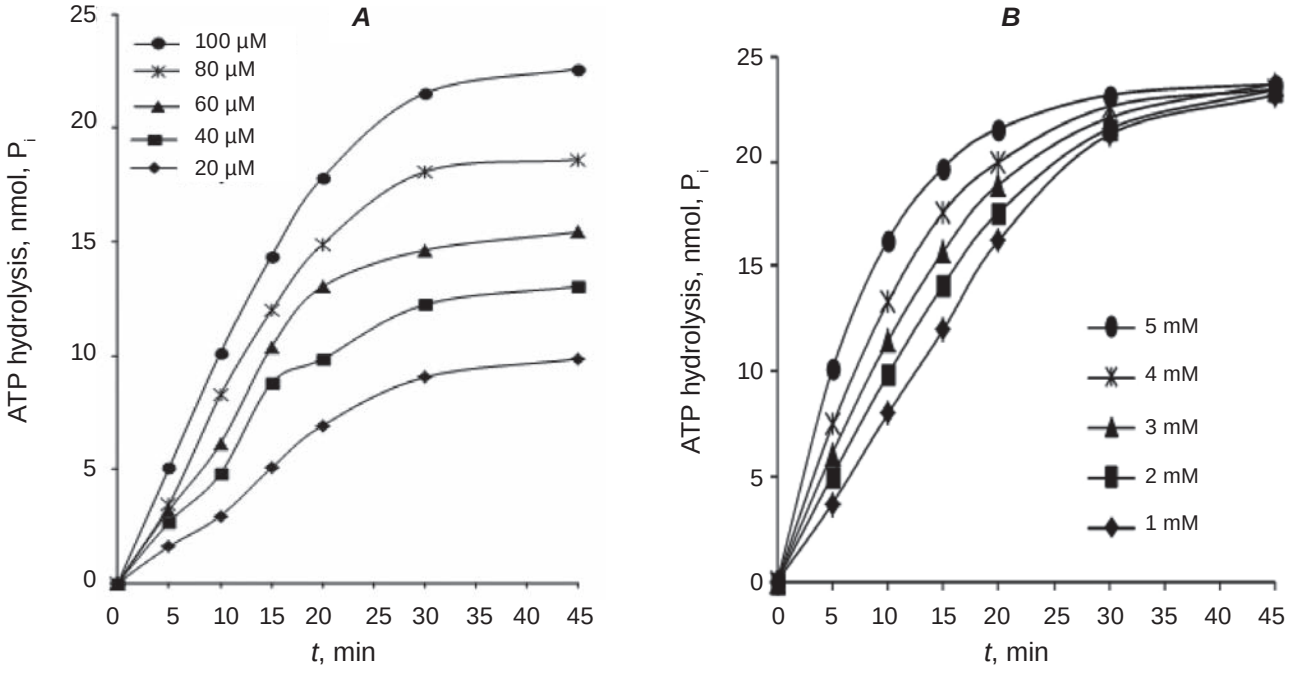

*Fig. 3. The concentration regularities of the effect of reagents on the kinetics of the ATP hydrolysis reaction, induced by calix[4]arene C-107 (the temperature 37°C, pH 7.4). Panel **A** – the effect of different concentrations of calix[4]arene, specified in the Figure (the ATP concentration was 1 mM), on the kinetics of the release of inorganic phosphate P_i. Panel **B** – the effect of different ATP concentrations, specified in the Figure (the concentration of calix[4]arene was 100 μM), on the kinetics of the release of inorganic phosphate P_i*

C-107. The abovedescribed kinetic analysis allows for approximate evaluation of the non-dimensional coefficient = k'/k, which characterizes the correlation between two rate constants on the level of the relevant stages of the transformation of calix[4]arene C-107 into non-reactive states (see scheme (1)) and for the determination of the individual values of these and other rate constants.

According to the ratio (12), we get

$$\gamma = (q_0/p_{max}) - 1. \quad (15)$$

Taking into consideration the fact that the volume of the incubation medium, in which calix[4]arene C-107 is available in the concentration of q_0 = 100 μM, is 0.4 ml and that the plateau value for the amount of the reaction product (inorganic phosphate P_i) p_{max} = 23 nmol (Fig. 2, panel *A*), for the maximal concentration of the reaction product that was formed (as per 1 L) during the time of reaction termination, we get the value of 57.5 μM. Thus, according to the equation (15), we get for the value γ: γ = (100 μM/57.5 μM) – 1 = 1.74 – 1 = 0.74.

According to the equation (11), in terms of the instant velocity *v* for ATP hydrolysis under the effect of calix[4]arene C-107, we can write:

$$v = dp/dt = k(\gamma+1)p_{max}s_0 \times \times e^{-k(\gamma+1)s0t/\{[(k'+k)/k1]+K+s0\}}/\{[(k'+k)/k1]+K+s0\}, \quad (16)$$

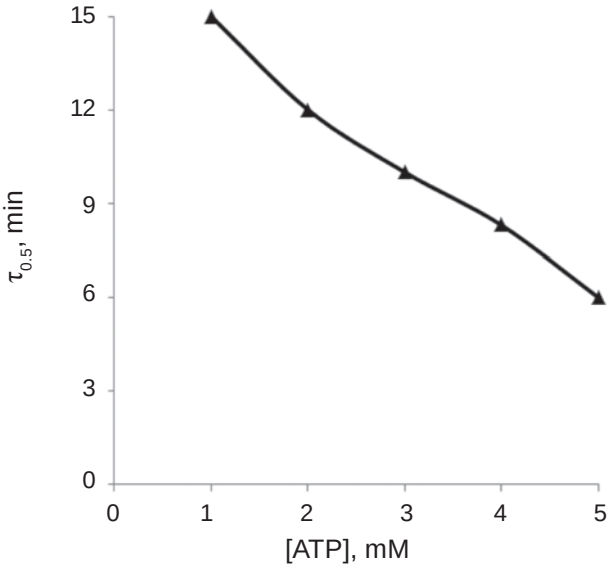

Fig. 4. The dependence of the period of half conversion of ATP under the effect of calix[4]arene C-107 (100 μM) $\tau_{0.5}$ on the concentration of nucleoside triphosphate s_0 (the temperature 37°C, pH 7.4)

from where in terms of the initial velocity of the reaction $v_0 = (dp/dt)_{t=0}$, we will have:

$$v_0 = v_{0,max}s_0/(<K>+s_0), \quad (17)$$

where $v_{0,max} = kq_0$ (18)

– the initial maximal velocity of the reaction of calixarene-induced hydrolysis of ATP, which depends exclusively on the total concentration of calix[4]arene, and

$$<K> = [(k'+k)/k_1]+K \quad (19)$$

– the additive imaginary constant, which, according to mechanism (1), unites all four constants of velocity (because the dissociation constant for the complex of calix[4]arene C-107 and ATP *K* equals the ratio of k_{-1}/k_1). According to the correlation (17), the value <*K*> numerically equals such total concentration s_0 of ATP, at which the initial velocity of the reaction v_0 corresponds to the value of 50% of the magnitude of the maximal velocity $v_{0,max}$, i.e. it equals $v_{0,max}/2$.

Thus, it is quite evident that the correlation (17) is identical to the main equation of the enzymatic kinetics – the classic equation of Michaelis–Menten. It should be noted that the imaginary constant <*K*> is identical to the Michaelis constant K_M in the enzymatic kinetics: as known, $K_M = k_{-1}/k_1 + k_{cat}/k_1 = K_s + k_{cat}/k_1$, where K_s – so-called substrate constant, characterizing the affinity of the substrate to the enzyme, and k_{cat} – a catalytic constant of the velocity of the enzymatic reaction; thus, in our case, the dissociation constant $K = k_{-1}/k_1$ of the intermediate complex of calix[4]arene C-107 and ATP QS (scheme 1) may be considered the analog of the substrate constant K_s in enzymology.

The chart of dependence of the initial velocity v_0 of the calixarene-induced ATP hydrolysis on the total concentration of ATP s_0 (concentration q_0 of calix[4]arene C-107 – 100 μM) is presented in Fig. 5, panel *A*. According to the form of the equation, linearized within Lineweaver-Burk coordinates (17) [$1/v_0$;$1/s_0$], we have a linear chart (r^2 = 0.93) (Fig. 5, panel *B*). Thus, the fact of this linearization directly demonstrates that the reaction of calix[4]arene-induced hydrolysis of ATP, which was studied in the mode of registering the initial velocity v_0, is evidently subject to the equation, similar to that of Michaelis-Menten (17).

Using the mentioned linear chart (Fig. 5, panel *B*), we can calculate the initial maximal velocity of the reaction $v_{0,max}$, and the imaginary constant <*K*>

(the analog of Michaelis' constant K_M in the kinetics of enzymatic reactions).

We see that $1/v_{0,max}$ = 0.423 (nmol P_i)$^{-1}$min^{-1}, then, $v_{0,max}$ = 2.4 nmol P_i/min. Taking into account the correlation (18), the value of the rate constant k regarding the decomposition of the "calixarene-ATP" QS complex (the analog of the constant of catalytic efficiency kcat in the kinetics of enzymatic reactions) towards the formation of the reaction product P_i will look as follows: $k = v_{0,max}/q_0$ = 2.4 nmol P_i min^{-1}/40 nmol P_i = 0.06 min^{-1} (because the value of the concentration of 100 μM calix[4]arene C-107 in the incubation medium of 0.4 ml corresponds to its amount which is equal to 40 nmol). However, the coefficient $\gamma = k'/k$ (see above) for the rate constant k' of the dissociation of the "calixarene-ATP" QS complex towards the formation of the reaction-incapable complex (QS)′ can be written as $k' = k\gamma$ = 0.06 min^{-1} 0.74 = 0.04 min^{-1}.

Thus, the abovepresented approximate evaluations demonstrate that the two stages, reflecting the transition of the "calixarene-C-107–ATP" complex into the reaction-incapable states in the course of the reaction – Q′ (with the rate constant k = 0.06 min^{-1}) and (QS)′ (with the rate constant k' = 0.04 min^{-1}), are implemented practically with the same probability for the mechanism of calix[4]arene-induced hydrolysis of ATP postulated by us (1).

The computer simulation of the "calix[4]arene C-107–ATP" QS complex was done in HyperChem 7.01. It was based on the fact that a molecule of calix[4]arene C-107 has two zwitterion fragments with protonated secondary amine nitrogen atoms and two deprotonated hydroxyls of phosphonate parts, and the ATP molecule is in a tetraanionic form. According to the results of the computer simulation of this complex, one can distinguish several structures characterized by the smallest total energies [2]. Thus, it is probable that the availability of two stages of the transition of QS complex into reaction-incapable states – Q′ and (QS)′ reflects the following notion: one of the structures of the QS complex is capable of ensuring the ATP hydrolysis (the rate constant k = 0.06 min^{-1}) with the simultaneous transition of calix[4]arene C-107 into reaction-incapable state Q′, and another structure of the complex QS is incapable of ensuring the ATP hydrolysis – this structure switches (the rate constant k' = 0.04 min^{-1}) into the reaction-incapable state (QS)′.

The evaluation of the values of the individual rate constants k_1 and k_{-1} in case of the stage of complex formation under the interaction between calix[4]arene C-107 and ATP. Based on the abovedescribed kinetic analysis, one can also evaluate the individual rate constants k_1 and k_{-1} at the

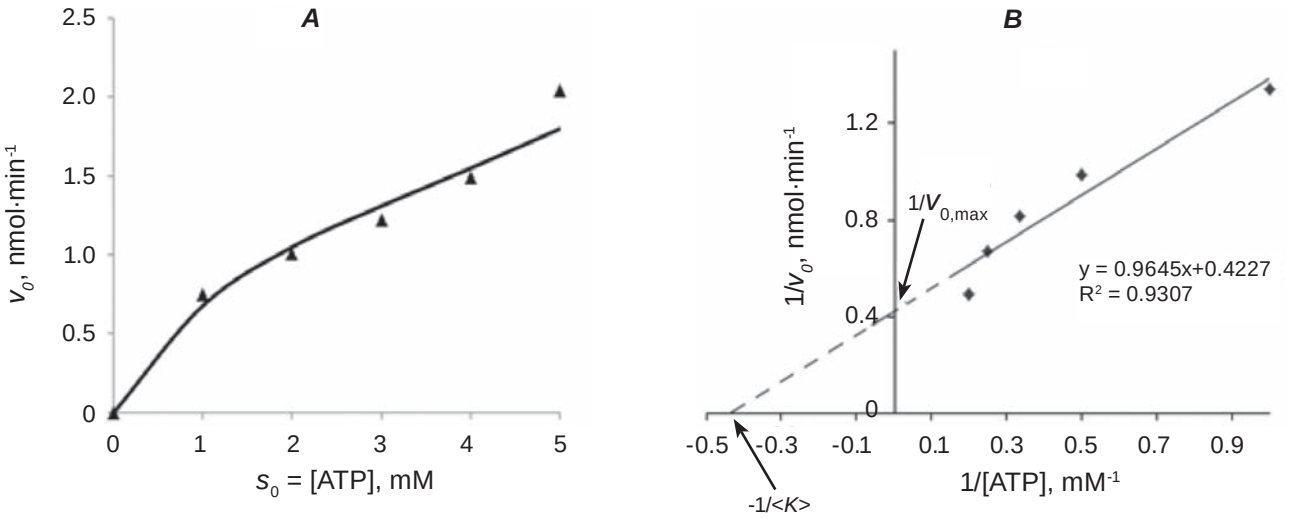

Fig. 5. The effect of the ATP concentration on the initial velocity v_0 of the reaction of hydrolysis of this nucleoside triphosphate ensured by calix[4]arene C-107 (100 μM) (the temperature 37°C, pH 7.4). Panel ***A*** *– the dependence of the initial velocity v_0 on the ATP concentration. Panel* ***B*** *– the Lineweaver-Burk plot for the dependence presented in Fig. 4, panel A (r^2 = 0.93). The values of the characteristic constants are as follows: $v_{0,max} = kq_0$ = 2.4 nmol P_i/min (k = 0.06 min^{-1}), <K> = $[(k'+k)/k_1]+K$ = 2.283 mM*

level of the stage of forming the intermediate "calix[4]arene C-107–ATP" QS complex (see scheme (1)). Indeed, using the linearized chart – the analog of Lineweaver-Burk chart (Fig. 5, panel *B*), we see that $-(1/\langle K\rangle) = -0.438$ mM^{-1}, i.e. $\langle K\rangle = 2.283$ mM, then, taking into account the correlation (19), the value of $(k'+k)/k_1$ will look as follows: $(k'+k)/k_1 = \langle K\rangle - K = 2.283$ mM – 0.215 mM = 2.068 mM. From here we will get the constant of the velocity for the association process k_1 in case of forming the QS complex: $k_1 = (k'+k)/(\langle K\rangle - K) = (0.04$ min^{-1} + 0.06 min$^{-1})/2.068$ mM = 0.05 mM^{-1}min^{-1} = 50 M^{-1}min^{-1}. However, considering the formula (5), we can write the following equation for the rate constant of the dissociation process k_{-1} of the QS complex towards the release of free calix[4]arene: $k_{-1} = k_1 K = 0.05$ mM^{-1}min^{-1}0.215 mM = 0.01 min^{-1}.

Therefore, it is easy to see that the ratio between the rate constants of the first order $(k/k_{-1}) = 0.06$ min^{-1}/0.01 min^{-1} = 6.0, i.e. in case of mechanism (1), the velocity of the implementation for the stage of calixarene-induced splitting of P_i exceeds considerably (almost six times) the velocity of dissociation of the calixarene-ATP complex with release of free calixarene.

The Table below summarizes the calculated values of the kinetic parameters of the ATP hydrolysis reaction induced by calix[4]arene C-107.

The identification of possible partial cases of the postulated mechanism of the ATP hydrolysis reaction, ensured by calix[4]arene C-107. As demonstrated above, the obtained experimental data regarding the kinetic regularities of the course of the ATP hydrolysis reaction, induced by calix[4]arene C-107 (Fig. 1–4), find their quite reliable explanation within the scheme (1). However, generally speaking, a partial case of the mechanism (1) of calix[4]arene-induced hydrolysis of ATP could be the one during which the "calix[4]arene C-107–ATP" (QS)′ complex is not formed at all, namely

$$Q + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} QS \xrightarrow{k} Q' + P. \qquad (20)$$

For this case we have: $k' = 0$, so the coefficient $\gamma = 0$. It means that under these conditions, according to the correlation (12), the plateau value (time-wise) for the amount of the reaction product pmax should equal the initial amount of calix[4]arene C-107 – 40 nmol (the initial concentration of calix[4]arene in the incubation medium – 100 μM, its volume – 40 μl). However, the typical plateau values p_{max} of the amount of inorganic phosphate P_i, registered by us in the experiments, were (depending on the total concentration of calix[4]arene q_0) 10–25 nmol, which is almost twice less than the value of the initial amount of calix[4]arene – 40 nmol. These calculations prove that the process of ATP hydrolysis under the effect of calix[4]arene C-107 takes place by the general scheme (1), not by the scheme (20).

Yet, theoretically there could be another variant of the mechanism (1), in which the coefficient $\gamma \gg 1$, i.e. according to the correlation (12), $k' \gg k$. It would mean that the velocity of transforming the reaction-capable complex QS into the reaction-incapable complex (QS)′ exceeds the velocity of forming the "inactivated" calix[4]arene Q′ and the reaction product P considerably. In this case, the plateau value pmax of the amount of the reaction product – inorganic phosphate P_i, will be determined by the equation: $p_{max} = (q_0/\gamma) \rightarrow 0$, i.e. the yield of the reaction product would be extremely low or practically absent which does not correspond to the experiment results (Fig. 2, panel *A*; Fig. 3).

Regarding some similarity between the reaction of ATP hydrolysis, induced by calix[4]arene C-107, and enzymatic reactions. Noteworthy is the following aspect: it comes to notice that from the standpoint of kinetic and energetic interpretation of the mechanism of the calixarene-induced ATP hydrolysis according to the scheme (1), there is some similarity between this reaction and a classic enzymatic reaction.

Firstly, an intermediate reaction-capable complex is identified both in the first and second cases – a complex of calix[4]arene with ATP QS and the enzyme-substrate complex, respectively.

Secondly, we also see the analogy in kinetic regularities of the course of the calixarene-induced hydrolysis of ATP and the classic enzymatic reaction while studying them in the mode of the initial velocity registration. Indeed, in the first and second cases, the dependence of the initial velocity v_0 on the concentration of reagents is described by the equation, similar to the canonic equation of Michaelis-Menten.

Thirdly, the value of the imaginary constant $\langle K\rangle \sim 10^{-3}$ M in case of the reaction of calixarene-induced hydrolysis of ATP on average and in some approximation fits into the range of typical values of Michaelis constants $K_M \sim 10^{-2}$–10^{-7} M, notable for enzymatic reactions (for the investigated enzymes, the density of the distribution of Michaelis constant is maximal at the value of $K_M \sim 10^{-4}$ M) [22-24].

Fourthly, there is some correlation between the energetics of the reaction of calixarene-induced hydrolysis of ATP (scheme (1)) and the energetics of enzymatic reactions. As stated above, according to our data, the value of the activation energy E_a for the reaction of calixarene-induced hydrolysis of ATP is 50.7 + 8.9 kJ/mol (see also [13]). As for the enzymatic reaction of ATP hydrolysis, catalyzed by the vector electroenzyme – Ca^{2+}-transporting Ca^{2+}, Mg^{2+}-ATPase of the plasma membrane, for instance, the value E_a is 56.4+1.5 kJ/mol [21] (it should be reminded that typical values E_a for the investigated enzymatic reactions on average correspond to the value of 20–80 kJ/mol [22]).

However, it is absolutely evident that there is no congruence in the comparison of the rate constant values k = 0.06 min^{-1} ~ 10^{-3} s^{-1} for the reaction of calixarene-induced ATP hydrolysis and the rate constant of the catalytic efficiency of enzymes k_{cat}, which is usually 1–10^5 s^{-1} for the investigated enzymes (the most common enzymes, which reactions are characterized by limiting stage with kcat values of approximately 10^2 s^{-1}) [22-24]. The efficiency of ATP transformation under the effect of calix[4]arene C-107 can be calculated as the ratio $k/\langle K\rangle$ = 10^{-3} s^{-1}/2.283 mM = 0.44 $M^{-1}s^{-1}$. The registered parameters of catalytic efficiency k_{cat}/K_M for enzymatic reactions are 10^1–10^{10} $M^{-1}s^{-1}$ (the most typical value ~ 10^5 $M^{-1}s^{-1}$) [22-24]. In other words, according to mechanism (1), the efficiency of ATP hydrolysis under the effect of calix[4]arene C-107 is many orders lower than the efficiency of transforming the substrates in case of enzymatic catalysis.

Thus, the mechanism (1) of ATP hydrolysis, induced by calix[4]arene C-107, suggested by us, is grounded on the idea that this reaction is accompanied by spontaneous transition of calix[4]arene from the state, capable of ensuring the hydrolytic process, into the state/states, in which calixarene cannot convert nucleoside triphosphate; i.e. it concerns some "inactivation" of calix[4]arene C-107 in the course of the mentioned reaction; the same process takes place for the reaction termination. In general, the abovepresented results give grounds for the assumption that the mechanism of the calixarene-induced hydrolysis of ATP, postulated by us at the level of a scheme (1), explains the kinetic properties of this reaction, which were determined in experimental studies, namely

– insignificant (< 10%) yield of the reaction product – inorganic phosphate Pi after the completion of the reaction (Fig. 2, panel *A*);

– successful description of the complete kinetic curve of the reaction product accumulation in the linearized format (Fig. 2);

– accurate quantitative description for the regularities of the plateau (time-wise) accumulation of the reaction product – inorganic phosphate P_i under conditions of the change in the concentration of ca-

T a b l e. The kinetic parameters of the ATP hydrolysis reaction, induced by calix[4]arene C-107 (scheme (1)). The temperature – 37°C, pH – 7.4

Kinetic parameter	Value of kinetic parameter
Coefficient γ	0.74
The first order rate constant k for the conversion of the "calix[4]arene-ATP" QS complex with formation of the product reaction P_i	0.06 min^{-1}
The first order rate constant k' for the conversion of the "calix[4]arene-ATP" QS complex with formation of the reaction-incapable complex (QS)′	0.04 min^{-1}
The second order rate constant k_1 for the association stage of the "calix[4]arene-ATP" complex formation	50 $M^{-1}min^{-1}$
The first order rate constant k_{-1} for the dissociation stage of the "calix[4]arene-ATP" complex towards the release of free calix[4]arene	0.01 min^{-1}
The initial maximal velocity of the reaction $v_{0,max}$	2.4 nmol P_i/min
The additive imaginary constant $\langle K\rangle$ that numerically equals the total concentration s_0 of ATP, at which the initial velocity of the reaction v_0 is $v_{0,max}/2$	2.3 mM
The efficiency of ATP transformation $k/\langle K\rangle$	0.44 $M^{-1}s^{-1}$

lix[4]arene (under stable ATP concentration) and a change in the ATP concentration (under stable concentration of calix[4]arene) (Fig. 3);

– reciprocal dependence of the period of half conversion of ATP under the effect of calix[4]arene on the concentration of nucleoside triphosphate (Fig. 4);

– the submission of the concentration dependence of the initial velocity v_0 of calix[4]arene-induced hydrolysis of ATP to Michaelis-Menten equation (Fig. 5).

The abovepresented kinetic analysis allowed for the quantitative interpretation of the ATP hydrolysis reaction (1), induced by calix[4]arene C-107, in terms of individual constants of velocity of its specific stages (Table).

However, one cannot rule out that the mentioned kinetic properties of the calixarene-induced hydrolysis of ATP (Fig. 2–5) may find their explanation within the sphere of application of other mechanism(s).

The experimental results presented in this article may be further developed to elaborate sensors, based on the application of calixarenes, to define ATP concentrations in biological liquids (blood, lymph, saliva, milk, colostrum, sperm, and urine). Indeed, firstly, as seen in Fig. 5, there is almost a linear reciprocal dependence between the value of the half conversion of ATP under the effect of calix[4]arene C-107 (100 μM) $\tau_{0.5}$ and the concentration of this nucleoside triphosphate s_0; in principle, this dependence may be interpreted as some calibration chart to determine the ATP concentration in the samples, containing biological liquids. Secondly, as already stated above, the physiologically significant bivalent cations Mg^{2+} and Ca^{2+}, which are immanently inherent to biological liquids, even in the concentration of 1 mM did not affect the velocity of the reaction of calixarene-induced hydrolysis of ATP; in addition, the physiologically significant monovalent cations Na^+ and K^+ in the concentration of 150 mM (which corresponds to the value of the ionic force for biological liquids) did not affect this reaction either [13]. Therefore, it is absolutely obvious that both abovementioned bivalent and monovalent cations will not interfere with the determination of the ATP concentration in biological liquids in case of using a calixarene-based sensor system. In our opinion, while using the targeted synthesis of new calix[4]arenes – analogs of calix[4]arene C-107, which will have higher affinity to ATP (equilibrium constant *K*) and higher velocity of hydrolysis of this nucleoside triphosphate (rate constant *k*), as compared to calix[4]arene C-107 in future, the issue of elaborating a sensor based on calix[4]arenes will become quite plausible.

It should be noted that the mechanism (1) of non-enzymatic calix[4]arene-induced ATP hydrolysis, presented in the article, is hypothetical, although it describes the experimental data exceptionally thoroughly (Fig. 3–5, Table). It means that other mechanisms, different from the suggested one (1), are quite possible too and can explain kinetic regularities in the course of the mentioned reaction. However, the final solution to the issue regarding the mechanism of calix[4]arene-induced ATP hydrolysis requires further experimental and theoretical studies, including stereochemical, aggregation-related and other aspects.

The promising fields are also the study of the kinetics, energetics, and mechanism of reactions of calixarene-induced non-enzymatic and calixarene-sensitive enzymatic hydrolysis of this nucleoside triphosphate, the elaboration of approaches to the calixarene-targeted modulation of the activity of cation-transporting and "mechanochemical" ATP-hydrolases. It can be expected that the data, accumulated in these studies, will allow for obtaining new information about the mechanism of non-enzymatic hydrolysis of ATP and the enzymatic ATP hydrolysis, related to active transport of cations.

Conflct of interest. Authors have completed the Unified Conflicts of Interest form at http://ukrbiochemjournal.org/wp-content/uploads/2018/12/coi_disclosure.pdf and declare no conflict of interest.

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КІНЕТИЧНІ ЗАКОНОМІРНОСТІ ТА МОЖЛИВИЙ МЕХАНІЗМ НЕЕНЗИМАТИЧНОГО ГІДРОЛІЗУ АТР ІНДУКОВАНОГО КАЛІКС[4] АРЕНОМ С-107

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Розроблено кінетичну модель калікс[4]арен-індукованого гідролізу АТР. Передбачається, що молекули калікс[4]арена С-107 утворюють комплекс із нуклеозидтрифосфатом, забезпечуючи виділення неорганічного фосфату P_i, а потім переходять у неактивний стан. Неактивні молекули калікс[4]арена вже не здатні утворювати комплекс з АТР і, відповідно, забезпечувати гідроліз нуклеозидтрифосфату. В експериментальних авторських дослідженнях вдалося пояснити кінетичні властивості реакції, а саме: незначний вихід кінцевого продукту реакції P_i; кількісні закономірності плато (у часі) накопичення продукту реакції при зміні концентрації калікс[4]арену С-107 або АТР; зворотна залежність напівперетворення АТР від його концентрації; відповідність початкової швидкості реакції від концентрації калікс[4]арена та АТР рівнянню Міхаеліса-Ментена. Остаточне рішення щодо молекулярного механізму калікс[4]арен-індукованого гідролізу АТР потребує подальших експериментальних і теоретичних досліджень.

Ключові слова: гідроліз АТР, каліксарени, емпіричний кінетичний аналіз.

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