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Kinetics of Ampicillin Synthesis Catalyzed by Penicillin Acylase from *E. coli* in Homogeneous and Heterogeneous Systems. Quantitative Characterization of Nucleophile Reactivity and Mathematical Modeling of the Process

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Abstract—Kinetic regularities of the enzymatic acyl group transfer reactions have been studied using ampicillin synthesis catalyzed by *E. coli* penicillin acylase as an example. It was shown that ampicillin synthesis proceeds through the formation of an acylenzyme–nucleophile complex capable of undergoing hydrolysis. The relative nucleophile reactivity of 6-aminopenicillanic acid (6-APA) is a complex parameter dependent on the nucleophile concentration. The kinetic analysis showed that the maximum yield of antibiotic being synthesized depended only on the nucleophile reactivity of 6-APA, the ratio between the enzyme reactivities with respect to the target product and acyl donor, and the initial concentrations of reagents. The parameters characterizing the nucleophile reactivity of 6-APA have been determined. The algorithm of modeling the enzymatic synthesis has been elaborated. The proposed algorithm allows the kinetics of the process not only in homogeneous, but also in heterogeneous ("aqueous solution–precipitate") systems to be quantitatively predicted and described based on experimental values of parameters of the reaction. It was shown that in heterogeneous "aqueous solution–precipitate" systems PA-catalyzed ampicillin synthesis proceeds much more efficiently compared to the homogeneous solution.

Key words: penicillin acylase, ampicillin synthesis, kinetic characteristics, nucleophile reactivity of 6-APA, mathematical modeling

Interest in the application of biocatalytic processes in organic synthesis has increased in recent years. One of the prominent examples of successful practical application of enzymes is the production of semi-synthetic β-lactam antibiotics with the aid of penicillin acylase (PA). Considerable advances have been made in this area in recent decades [1-4]. Taking into account the practical interest in this problem, the study of the corresponding reaction mechanisms has acquired importance because knowledge of such mechanisms allow the general features of the synthesis of β-lactam antibiotics to be explained and efficient methods for optimization of their synthesis to be elaborated.

It has been well documented [5-7] that the reactions catalyzed by PA proceed through the formation of an acylenzyme intermediate. This is shown in particular by inactivation of PA by phenylmethylsulfonyl fluoride (PMSF) and its analogs modifying catalytically active B1 serine, and partial reactivation of the inactivated enzyme by external nucleophile [8]. Recent data of X-ray analysis of the complex of PA with PMSF support these suggestions [9]. To explain the regularities of the PA-catalyzed acyl transfer reactions to the nucleophile, several kinetic schemes (Schemes 1 and 2) have been proposed. According to the simplest scheme (Scheme 1) [6], the reaction proceeds through the formation of an acylenzyme, which may be directly attacked by a nucleophile yielding the target antibiotic (P) or by water (acting as a competing nucleophile) giving a by-product of hydrolysis P_2. However, the investigations carried out in [5, 7, 10, 11] showed that experimental data obtained could not be explained by Scheme 1, and synthesis supposedly proceeded with the formation an intermediate acylenzyme–nucleophile complex, which in turn gave rise to the target product or underwent hydrolysis giving the

Abbreviations: PA) penicillin acylase; 6-APA) 6-aminopenicillanic acid; D-PG) D-phenylglycine; D-PGME) D-phenylglycine methyl ester; PMSF) phenylmethylsulfonyl fluoride.

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by-product P_2 (Scheme 2). Kinetic analysis of the enzymatic acyl group transfer reactions [12, 13] showed that the maximum concentration of the product of synthesis[1] depended solely on the values of two complex kinetic parameters: the relative reactivity of the nucleophile and a specificity parameter (the ratio of the k_{cat}/K_m values for hydrolysis of acyl donor and the target product) as well as on the initial concentrations of the acyl donor and the nucleophile. This conclusion is true both for Scheme 1 and the more complex Scheme 2. Unfortunately, there is no experimental data in the literature that would make it possible to carry out the quantitative analysis of the kinetic regularities of the enzymatic synthesis of β-lactam antibiotics in the required way. Therefore, in the present paper we report results of our studies on the kinetic characterization of the enzymatic acyl transfer reactions to nucleophile using PA-catalyzed ampicillin synthesis as an example and elaboration of the kinetic model for adequate description of the experimental data over a wide range of the conditions (from homogeneous solutions to heterogeneous systems "aqueous solution–precipitate").

MATERIALS AND METHODS

Reagents. The PA preparation was purified from *E. coli* ATCC 9637 by a method developed by us previously [14]. The concentration of active sites in the purified preparation was determined by titration of the enzyme with PMSF [8] and found to be $3 \cdot 10^{-5}$ M. 6-APA, ampicillin, and D-phenylglycine methyl ester (D-PGME) were donated by DSM (The Netherlands), and D-phenylglycine (D-PG) was purchased from Sigma (USA). Other reagents and the components of the buffer systems were from Merck (Germany). Organic solvents (extra high purity) were from Russian suppliers.

Study of the dependence of nucleophile reactivity of 6-APA on the concentration of the acyl donor. We define the nucleophile reactivity of 6-APA as its relative reactivity in the reaction of enzymatic transfer of an acyl moiety of the substrate to 6-APA and water as a competing nucleophile. Parameter β in Eq. (13), which is the ratio of the initial accumulation rates of ampicillin and D-PG, is taken as a quantitative measure of the nucleophile reactivity. To study the dependence of the nucleophile reactivity of 6-APA on the concentration of the acyl donor (D-PGME), we measured the initial accumulation rates of ampicillin and D-PG at fixed 6-APA concentration (50 mM); D-PGME concentration was varied from 20 to 200 mM. The enzymatic reactions were carried out in a thermostatted cell of a pH-stat (Radiometer RTS-622, Denmark) with constant stirring. Specified amounts of reagents were added to 10 ml of water, and the mixtures (pH 6.3) were incubated at 25°C for 10 min. The reaction was initiated by the addition of 100 μl of the enzyme solution. The pH value was held constant by automatic titration. Aliquots (20-50 μl) were withdrawn in the course of the reaction and diluted by a corresponding volume (980-950 μl) of eluent to terminate the enzymatic process. Samples obtained were analyzed by HPLC using a device consisting of an Altex 110A pump, a 4.6 × 150 mm Nucleosil C-18 (Chrompack) reverse-phase column, an LKB 2138 S detector (with registration at 214 nm), and an LKB 2221 integrator. The eluent containing 30% (v/v) acetonitrile, ion-pair reagent SDS (0.68 g/liter), and 5 mM phosphate (pH 3.0) were used. The rate of elution was 1.0 ml/min.

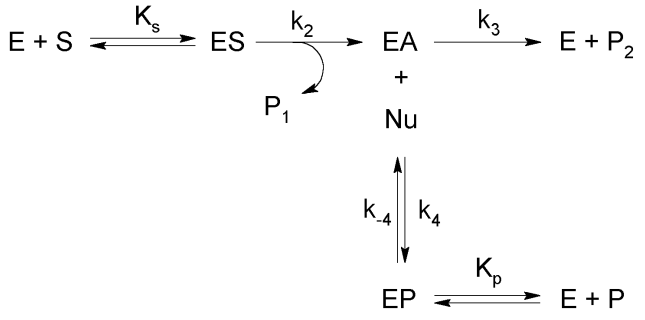
Determination of parameters of 6-APA nucleophile reactivity in the reaction of enzymatic synthesis of ampicillin. Parameters of the reactivity of 6-APA, β_0 and γ in Eq. (13), were determined by analyzing the dependence of the ratio of the initial accumulation rates of ampicillin and D-PG in the course of enzymatic synthesis using 100 mM D-PGME as acyl donor on 6-APA concentration (in the range from 20 to 200 mM). The reactions were carried on in the thermostatted cell of the pH-stat with constant stirring, as described above.

Determination of the kinetic parameters of the enzymatic hydrolysis of D-PGME and ampicillin. The kinetic parameters of the enzymatic hydrolysis of ampicillin and D-PGME (k_{cat} and K_m) were determined based on analysis of the dependence of the initial rate of hydrolysis of the corresponding substrate on its concentration. The reactions of enzymatic hydrolysis were carried on in the thermostatted cell of the pH-stat with constant stirring, as described above. Aliquots (20-50 μl) were withdrawn in the course of the reaction, diluted with the corresponding volume of eluent (980-950 μl), and analyzed by HPLC.

Study of the enzymatic synthesis of ampicillin in homogeneous and heterogeneous systems. The reactions were carried out in the thermostatted cell of the pH-stat with constant stirring. The corresponding amounts of 6-APA (50-300 mM) and D-PGME (50-500 mM) were added to 10 ml of water. The reaction mixture (pH 6.3) was incubated at 25°C for 10 min. The reaction was initiated by the addition of 700 μl of the enzyme solution. Aliquots withdrawn in the course of the reaction were analyzed by HPLC. In the case of heterogeneous systems, two aliquots were withdrawn simultaneously to analyze the composition of the reaction mixture. One aliquot was withdrawn using a Chromafil microfilter (Bester, The Netherlands) with 0.45-μm pore diameter to separate the precipitate and analyze the composition of the solution, whereas other aliquot was withdrawn directly from the heterogeneous mixture to characterize the whole system.

[1] The product accumulation curve in the enzymatic acyl group transfer reactions in aqueous media has a maximum [12], which corresponds to the maximum concentration of the target product.

Kinetic schemes and equations. Scheme 1 is the "minimum" kinetic scheme of the enzymatic acyl group transfer to nucleophile:



Scheme 1

Here E is the free enzyme, S is the activated acyl donor, P_1 is the first product being released during the formation of acylenzyme, Nu is nucleophile (antibiotic nucleus), P_2 is the product of hydrolysis of the acylenzyme, P is the product of acyl transfer to nucleophile (the target antibiotic), ES is the enzyme–substrate complex, EA is acylenzyme, and EP is the enzyme–product complex. The expressions for the rates of P and P_2 formation have the following form:

$$\frac{dp}{dt} = e\left[\frac{k_2k_4ns}{K_S(k_3+k_4n)} - \frac{k_3k_{-4}p}{K_P(k_3+k_4n)}\right]; \quad (1)$$

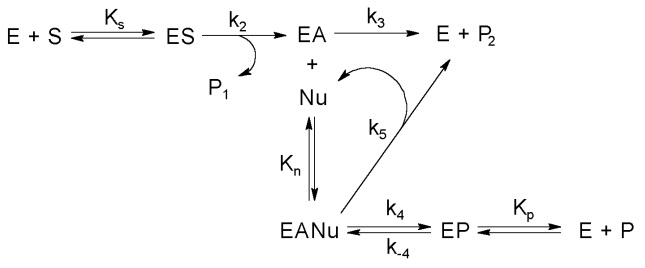
$$\frac{dp_2}{dt} = e\left[k_3\left(\frac{k_2s}{K_S(k_3+k_4n)} + \frac{k_{-4}p}{K_P(k_3+k_4n)}\right)\right]; \quad (2)$$

$$e = e_0\Big/\left[1 + \frac{s}{K_S} + \frac{p}{K_P} + \frac{k_2s}{K_S(k_3+k_4n)} + \frac{k_{-4}p}{K_P(k_3+k_4n)}\right]; \quad (3)$$

$$s_0 = s + p + p_2; \quad (4)$$

$$n_0 = n + p. \quad (5)$$

Scheme 2 shows the kinetics of the enzymatic acyl group transfer to nucleophile with intermediate formation of an acylenzyme–nucleophile complex:



Scheme 2

All the designations in Scheme 2 correspond to those given for Scheme 1. The additional designation (EANu) refers to the acylenzyme–nucleophile complex, which is converted into the product P or hydrolyzed with the formation of the product P_2. The kinetic equations are transformed as follows:

$$\frac{dp}{dt} = e\left[\frac{k_2k_4ns}{K_S(k_3K_n+k_4n+k_5n)} - \frac{k_{-4}p(k_3K_n+k_5n)}{K_P(k_3K_n+k_4n+k_5n)}\right]; \quad (6)$$

$$\frac{dp_2}{dt} = e\left[(k_3K_n+k_5n)\left(\frac{k_2s}{K_S(k_3K_n+k_4n+k_5n)} + \frac{k_{-4}p}{K_P(k_3K_n+k_4n+k_5n)}\right)\right]; \quad (7)$$

$$e = e_0\Big/\left[1 + \frac{s}{K_S} + \frac{p}{K_P} + \frac{k_2s(K_n+n)}{K_S(k_3K_n+k_4n+k_5n)} + \frac{k_{-4}p(K_n+n)}{K_P(k_3K_n+k_4n+k_5n)}\right]; \quad (8)$$

$$s_0 = s + p + p_2; \quad (9)$$

$$n_0 = n + p. \quad (10)$$

The systems of equations describing kinetic Scheme 1 (Eqs. (1)-(5)) and Scheme 2 (Eqs. (6)-(10)) were obtained using the assumption of a steady state for EA and EANu. Here and in the following equations, the current concentrations of the substances are given by small letters corresponding to capital letters in the kinetic schemes. Subscript "0" is used for designation of the initial concentration, and t is time. The initial conditions, unless otherwise specified, are the following: $t = 0$, $s = s_0$, $n = n_0$, and $p_2 = p = 0$.

RESULTS AND DISCUSSION

Choice of the Adequate Kinetic Scheme

To establish the "minimum" kinetic scheme adequately describing the PA-catalyzed acyl group transfer to 6-APA, we studied the dependence of the nucleophile reactivity of 6-APA on the concentrations of acyl donor and antibiotic nucleus. The analysis of kinetic Schemes 1 and 2 results in the following expressions for the ratio of the initial rates of ampicillin accumulation (the rate of synthesis, v_{amp}) and D-PG (the rate of hydrolysis, $v_{D\text{-}PG}$):

for Scheme 1:
$$\left(\frac{V_{amp}}{V_{D\text{-}PG}}\right)_{init} = \frac{k_4n}{k_3}; \quad (11)$$

for Scheme 2:
$$\left(\frac{V_{amp}}{V_{D\text{-}PG}}\right)_{init} = \frac{k_4n}{k_3K_n + k_5n}. \quad (12)$$

Thus, analysis of the dependence of the ratio of the initial ampicillin and D-PG accumulation rates on the concentration of nucleophile allows the formation of the acylenzyme–nucleophile complex undergoing hydrolysis with rate constant k_5 (Scheme 2) to be found. For the sake of convenience, introduce the following designations in Eq. (12): $\beta_0 = k_4/k_3K_n$ (parameter characterizing the relative reactivity of 6-APA at low nucleophile concentrations, i.e., at $n_0 << k_3K_n/k_5$) and $\gamma = k_5/k_4$ (parameter characterizing the ratio of the hydrolysis and synthesis rates at the conversion of the acylenzyme–nucleophile complex). In such event, parameter β in Scheme 2 characterizes quantitatively the efficiency of acyltransfer to nucleophile, i.e., the relative reactivity of 6-APA at certain concentration of the latter:

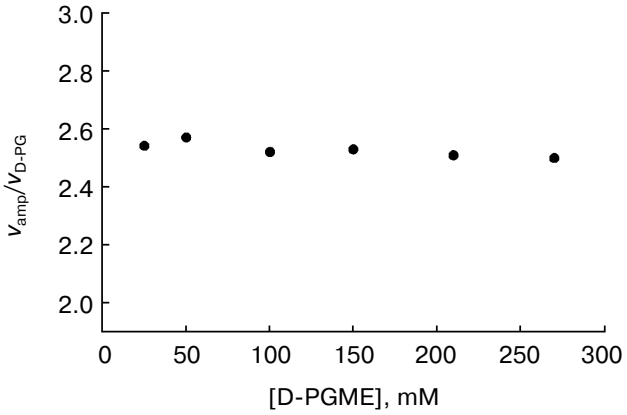


Fig. 1. Dependence of the ratio of initial rates of accumulation of ampicillin and D-PG on the concentration of donor of acyl moiety (D-PGME). Conditions: 50 mM 6-APA, pH 6.3; 25°C.

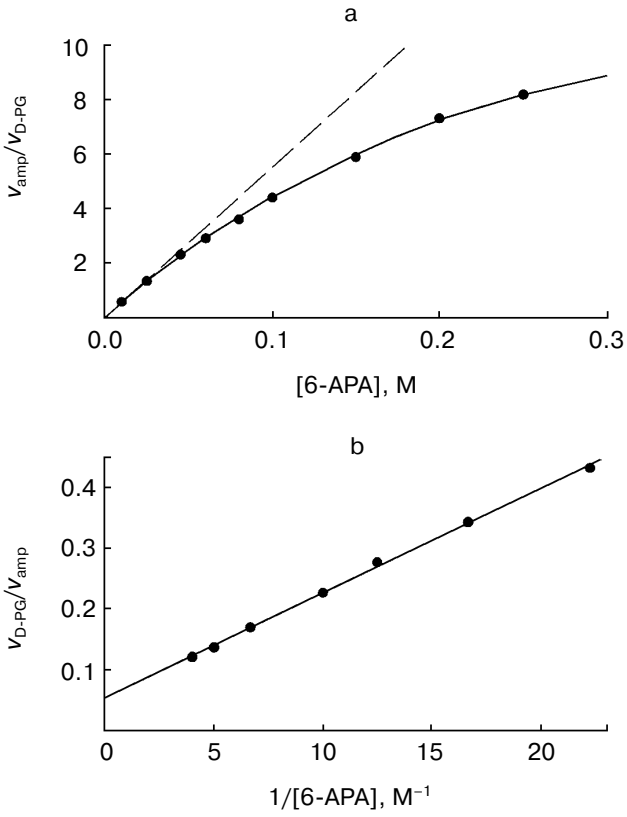


Fig. 2. Dependence of the ratio of initial ampicillin and D-PG accumulation rates on 6-APA concentration (a) and the linear anamorphosis in the reciprocal coordinates (b). The dotted line corresponds to the hypothetical situation when $k_5 = 0$. Conditions: 100 mM D-PGME, pH 6.3; 25°C.

$$\left(\frac{V_{amp}}{V_{D\text{-}PG}}\right)_{init} = \beta n\ , \qquad \text{where}\ \ \beta = \frac{\beta_0}{1+\beta_0\gamma n}\ . \tag{13}$$

Thus, when the enzymatic acyl transfer reaction proceeds without the formation of the acylenzyme–nucleophile complex capable of giving the by-product P_2 due to hydrolysis (Scheme 1), the ratio of the synthesis and hydrolysis rates should rise linearly as the concentration of nucleophile increases (Eq. (11)). For the scheme involving the formation of the EANu complex (Scheme 2; $k_5 > 0$), the dependence under discussion takes the form of a curve with saturation (Eq. (13)). Our investigations showed that the nucleophile reactivity of 6-APA was independent of the concentration of the acyl donor (Fig. 1), suggesting that both reactions (synthesis and hydrolysis) proceed through the formation of a common acylenzyme intermediate. As for the dependence of the ratio of the initial synthesis and hydrolysis rates on 6-APA concentration, a curve with saturation was obtained (Fig. 2a). We conclude that kinetic Scheme 2 is the "minimum" kinetic scheme that allows the experimental data to be adequately described. In the following, this scheme will be used for elaborating the mathematical model of the enzymatic synthesis of ampicillin. The values of the nucleophile reactivity of 6-APA estimated from parameters β_0 and γ (Fig. 3) are comparable with the corresponding ratios of the rate constants for acyl transfer to nucleophile and water (k_4/k_3) determined for the reactions catalyzed by other hydrolases, such as α-chymotrypsin (k_4/k_3 = 50-300 M^{-1} [15]) or trypsin (k_4/k_3 = 30-50 M^{-1} [16]). However, it should be noted that in the works cited [15, 16] the nucleophile reactivity was determined assuming the reaction followed Scheme 1, ignoring the possibility of the formation of the acylenzyme–nucleophile complex. This circumstance gives no way of describing the reactions at high concentrations of nucleophile.

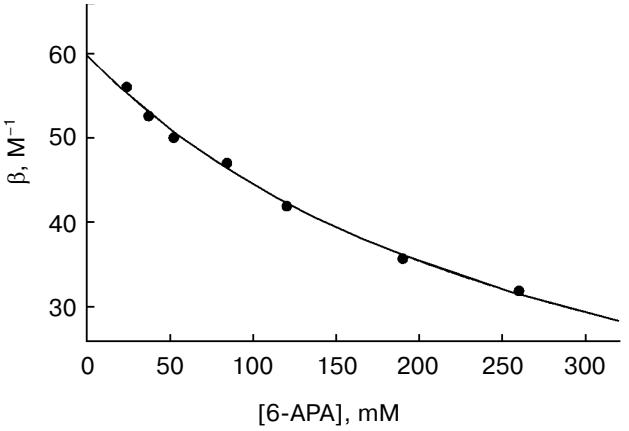


Fig. 3. Dependence of nucleophile reactivity of 6-APA (parameter β) in the PA-catalyzed ampicillin synthesis on 6-APA concentration. Parameters of the nucleophile reactivity: $\beta_0 = 60$ M^{-1} and $\gamma = 0.056$. Conditions: 100 mM D-PGME, pH 6.3; 25°C.

Analysis of the Model

Determination of kinetic parameters of the model. Taking into account the expressions determining the nucleophile reactivity of 6-APA (parameters β_0 and γ), Eqs. (6)-(10) can be transformed as follows:

$$\frac{dp}{dt} = e\frac{k_2}{K_S}\frac{\beta_0 ns - \alpha p(1+\beta_0\gamma n)}{1+\beta_0 n+\beta_0\gamma n}\ ; \qquad (14)$$

$$\frac{dp_2}{dt} = e\frac{k_2}{K_S}\frac{(1+\beta_0\gamma n)(s+\alpha p)}{1+\beta_0 n+\beta_0\gamma n}\ ; \qquad (15)$$

$$e = e_0\Bigg/\left(1+\frac{s}{K_S}+\frac{p}{K_P}+\frac{k_2}{K_S}\left(\frac{(s+\alpha p)(n+K_n)\beta_0}{k_4(1+\beta_0 n+\beta_0\gamma n)}\right)\right); \qquad (16)$$

$$s_0 = s + p + p_2\ ; \qquad (17)$$

$$n_0 = n + p\ , \qquad (18)$$

where parameter $\alpha = (k_{-4}/K_P)/(k_2/K_S)$ is the ratio of the second-order rate constants for the enzymatic hydrolysis of the target product and acyl donor under the reaction conditions. This parameter characterizes the specificity of PA with respect to these two substrates. It is obvious that the solution of the system of equations (14)-(18) allows the current concentrations of all components of the reaction to be calculated at every instant and, consequently, the kinetic characteristics of the biocatalytic synthesis of ampicillin to be analyzed. The model was quantitatively analyzed by numerical solution of the system of equations (14)-(18) using the Runge–Kutta fourth-order method. The computer program Mathcad 7.0 was applied in these calculations. Parameters of the nucleophile reactivity of 6-APA (β_0 and γ) were estimated by analyzing the dependence of the ratio of the initial ampicillin and D-PG accumulation rates on the concentration of 6-APA, as shown in Fig. 2. The parameters (k_{cat} and K_m) of hydrolysis of the substrates, D-PGME and ampicillin, were determined in a separate experiment from the dependence of the initial rate of the enzymatic hydrolysis of a certain substrate on its initial concentration. Based on the experimental data (Table 1), the individual kinetic and equilibrium constants needed for modeling were calculated (Table 2).

Effect of kinetic parameters and initial concentrations of reagents on the maximum efficiency of ampicillin synthesis. Analysis of Eq. (14) shows that the ampicillin accumulation curve passes through a maximum. The condition for appearance of a maximum is $dp/dt = 0$ or

$$\alpha p^* + \alpha\beta_0\gamma p^*(n_0 - p^*) - \beta(n_0 - p^*)(s_0 - p^* - p_2^*) = 0, \qquad (19)$$

where p^* and p_2^* are the concentrations of ampicillin and D-PG at the maximum point. It follows from the equations describing the accumulation of the products (Eqs. (14) and (15)) and material balance equations (Eqs. (17) and (18)) that:

$$p_2 = f(\alpha, \beta, \gamma, n_0, s_0, p). \qquad (20)$$

Thus, the efficiency of synthesis (the maximum concentration of ampicillin) is independent on the absolute values of the individual constants but is determined solely by the values of parameters α, β_0, and γ and the initial concentrations of acyl donor and nucleophile. The solution of Eq. (19) in analytical form is lacking. However, if we assume that the rates of product accumulation remain constant up to the time corresponding to the maximum point, Eq. (20) can be written as follows:

$$p_2^* = \gamma p^* + \frac{1}{\beta}\ln\frac{n_0}{n_0 - p^*}\ .$$

Then we expand a logarithm in series and substitute the expression obtained in Eq. (19). If we restrict ourselves to the first term of the expansion (this is true at $p^* << n_0$), the quadratic equation for maximum of ampicillin accumulation can be obtained. The equation has the following solution:

$$p^* = \frac{n_0}{2d}\left(b - \sqrt{b^2 - \frac{4dc}{n_0}}\right), \qquad (21)$$

Table 1. Experimental values of parameters of enzymatic synthesis of ampicillin catalyzed by PA with D-PGME as a donor (pH 6.3; 25°C)

$\beta_0 = \frac{k_4}{k_3 K_n}$, M^{-1}	$\gamma = \frac{k_5}{k_4}$	$k_{cat}^{D\text{-}PGME} = \frac{k_2 k_3}{k_2 + k_3}$, sec^{-1}	$k_{cat}^{amp} = \frac{k_{-4} k_3}{k_{-4} + k_3}$, sec^{-1}	$\left(\frac{k_{cat}}{K_M}\right)^{D\text{-}PGME} = \frac{k_2}{K_S}$, $M^{-1} \cdot sec^{-1}$	$\left(\frac{k_{cat}}{K_M}\right)^{amp} = \frac{k_{-4}}{K_P}$, $M^{-1} \cdot sec^{-1}$
60	0.056	30	25	430	980

Table 2. Values of the kinetic and equilibrium constants used for modeling the enzymatic synthesis of ampicillin*

k_2, sec^{-1}	k_{-4}, sec^{-1}	k_3, sec^{-1}	k_4, sec^{-1}	k_5, sec^{-1}	K_s, M	K_p, M	K_n, M
100	50	50	150	7.5	0.23	0.05	0.05

* Absolute values of the kinetic and equilibrium constants were calculated on the basis of the experimental values of parameters given in Table 1 and optimized with the computer program Mathcad 7.0 for best agreement between the experimental data and the computed curves of accumulation of the products of the reaction.

where $d = 1 + \beta_0 n_0 + \beta_0 \gamma n_0 + \alpha\beta_0 \gamma n_0$, $b = d + \beta s_0 + \alpha$, $c = \beta s_0 n_0$.

The second root of the equation does not satisfy the conditions of material balance. The expression for the maximum concentration of the target product being synthesized, which was obtained using an expansion in series, is valid at $p^* << n_0$. However, since the p_2^* function is not a single parameter, which determines the p^* value (see Eq. (19)), the approximate equation (21) can be used over a wide range of conditions. Figure 4 shows the results of calculation of the maximum degree of conversion of 6-APA into ampicillin on varying the initial concentrations of acyl donor and nucleophile. The calculations were carried out based on Eq. (21) and numerical solution of the system of equations (14)-(18). It is obvious that analytical expression (21) predicts the maximum yield of the target product to a good approximation, especially at rather high concentrations of 6-APA, and can be used for estimation of the efficiency of synthesis over a wide range of the conditions.

The fact that the system of equations describing the kinetics of acyl group transfer to nucleophile may be solved in numerical form allows the effects of parameters α, β_0, and γ on the maximum yield of the target product to be analyzed. Figures 5 and 6 show the maximum degree of conversion of 6-APA to ampicillin ($F_{Nu} = p^*/n_0$) as a function of parameters α, β_0, and γ as well as concentrations n_0 and s_0. The values of F_{Nu} were calculated based on the numerical solution of the system of equations (14)-(18) using experimental values of the modeling parameters. It is obvious that the efficiency of synthesis increases with the nucleophile reactivity of 6-APA (increase in parameter β_0 and decrease in parameter γ) and increase in the reactivity of the acyl donor (decrease in parameter α). Besides, analysis of data presented in Fig. 6 shows that the use of the highest possible initial concentrations of acyl donor and nucleophile is a necessary condition for obtaining high yield in ampicillin synthesis. Using high concentrations of the reagents means that the reaction system is actu-

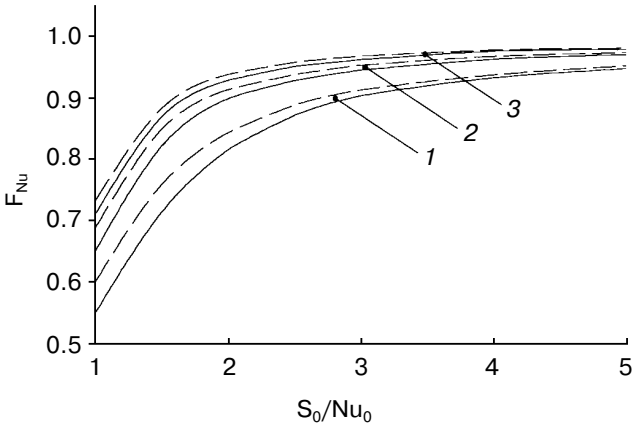


Fig. 4. Theoretical dependences of the maximum degree of 6-APA conversion into ampicillin (F_{Nu}) on the ratio of initial concentrations of nucleophile (6-APA) and acyl donor (D-PGME) calculated at various initial concentrations of nucleophile n_0 (M): 0.2 (*1*), 0.4 (*2*), and 0.6 (*3*). The dashed lines show the results of calculations with the approximate formula (21). The solid lines were obtained by numeric solution of the system of equations (14)-(18). Parameters of the model: $\alpha = 2$, $\beta_0 = 60$ M^{-1}, and $\gamma = 0.056$.

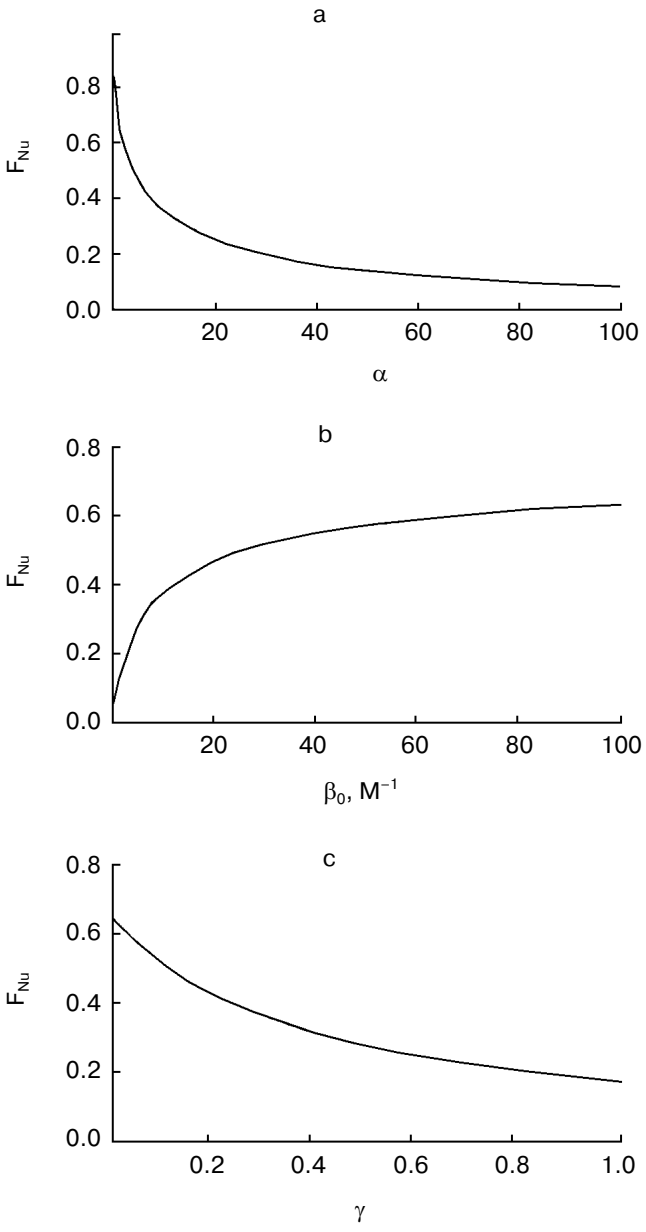


Fig. 5. Dependence of maximum degree of conversion of 6-APA into the target product (F_{Nu}) on values of parameters α (a), β_0 (b), and γ (c). Parameters of the model: $n_0 = 0.5$ M, $s_0 = 0.5$ M, $\alpha = 2$, $\beta_0 = 60$ M^{-1}, and $\gamma = 0.056$.

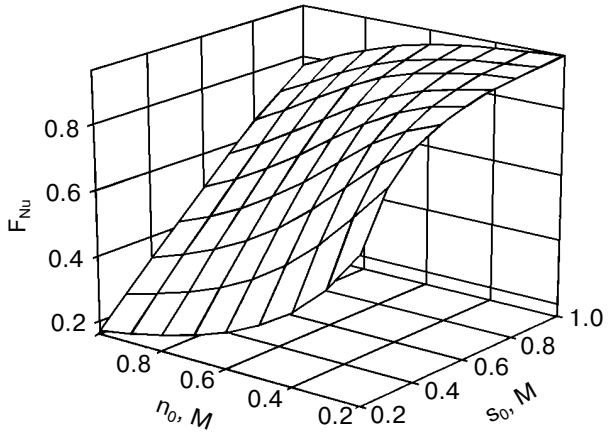


Fig. 6. Dependence of the maximum degree of conversion of 6-APA into the target product (F_{Nu}) on the initial concentrations of 6-APA and the donor of the acyl moiety (D-PGME). Parameters of the model: $\alpha = 2$, $\beta_0 = 60$ M^{-1}, and $\gamma = 0.056$.

ally a heterogeneous system, where one or several components are present as a precipitate, since the solubilities of the products of the reaction (ampicillin and D-PG) at a given value of pH are considerably lower than those of the starting substrates (see Table 3). Table 4 summarizes the experimental data for the enzymatic synthesis of ampicillin in systems with increasing concentrations of the original reagents (6-APA and D-PGME) as well as the results of calculations carried out on the basis of the numerical solution of Eqs. (14)-(18) using the values of parameters given in Table 2. Our mathematical model allows the efficiency of the enzymatic synthesis in homogeneous or heterogeneous systems containing only D-PG in the precipitate to be predicted to a good approximation. In the case of heterogeneous systems containing both D-PG and ampicillin in the precipitate, the kinetic regularities of the enzymatic synthesis in circumstances where the precipitate of the target product is being formed call for special consideration (see below).

Kinetic regularities of the enzymatic ampicillin synthesis in homogeneous and heterogeneous systems. The mathematical model of the enzymatic acyl group transfer to nucleophile allowed us not only to analyze the maximum value on the accumulation curve of the target product, but also to calculate the progress curves for all the components of the reaction. To study the kinetic characteristics of the enzymatic synthesis in homogeneous sys-

Table 3. Solubility of components of the reaction mixture where the enzymatic synthesis of ampicillin catalyzed by PA is being carried on (pH 6.3; 25°C)

Component of the reaction	Solubility, M
D-PGME	1.8
6-APA	0.28
Ampicillin	0.025
D-PG	0.035

Table 4. Experimental and computed values of the maximum degree of conversion of 6-APA into ampicillin during the enzymatic synthesis of ampicillin catalyzed by *E. coli* PA (pH 6.3; 25°C)

Original system	n_0/s_0, M	Maximum yield, %	
		experiment	calculation
Homogeneous systems	0.05 : 0.05	29	28
Heterogeneous systems with precipitate of D-PG	0.05 : 0.1	46	46
	0.1 : 0.32	75	76
Heterogeneous systems with precipitates of D-PG and ampicillin*	0.15 : 0.35	83	85
	0.28 : 0.5	87	88

* Calculations were carried out with regard for the formation of the precipitate of the target product as described in the text.

tems, the enzymatic synthesis of ampicillin from D-PGME and 6-APA at pH 6.3 and 25°C was modeled in various concentration regimes. As seen from Fig. 7, the results of modeling using Eqs. (14)-(18) and the values of parameters given in Table 2 describe the kinetics of ampicillin synthesis in the homogeneous systems (Fig. 7a) and heterogeneous systems where the precipitate contains only D-PG (Fig. 7b) to a good approximation. When synthesis of ampicillin is accompanied by the formation of a precipitate containing both D-PG and ampicillin (Fig. 8), substantial discrepancies between the experimental data and the results of modeling (curves *1* and *3*) are observed. The discrepancies are concerned not only with the shape of the product accumulation curves, but also with the maximum value of accumulation of the target product. To correctly describe such systems, one should take into account that the real concentration of ampicillin in the reaction system differs from its total concentration and is determined by the solubility of ampicillin as well as the effects of supersaturation. When a precipitate of product p is formed, the system of equations describing the acyl transfer to the antibiotic nucleus differs significantly from that for the homogeneous reaction system and has the following form:

$$\frac{dp}{dt} = e\frac{k_2}{K_S}\frac{\beta_0 ns - \alpha p_{\text{het}}\left(1+\beta_0\gamma n\right)}{1+\beta_0 n+\beta_0\gamma n}\ ; \qquad (22)$$

$$\frac{dp_2}{dt} = e\frac{k_2}{K_S}\frac{\left(1+\beta_0\gamma n\right)\left(s+\alpha p_{\text{het}}\right)}{1+\beta_0 n+\beta_0\gamma n}\ ; \qquad (23)$$

$$e = e_0\Bigg/\left(1+\frac{s}{K_S}+\frac{p_{\text{het}}}{K_P}+\frac{k_2}{K_S}\left(\frac{\left(s+\alpha p_{\text{het}}\right)\left(n+K_n\right)\beta_0}{k_4\left(1+\beta_0 n+\beta_0\gamma n\right)}\right)\right)\ ; \qquad (24)$$

$$s_0 = s + p + p_2\ ; \qquad (25)$$

$$n_0 = n + p\ , \qquad (26)$$

where p_{het} is the concentration of ampicillin in the solution. The exact form of the $p_{\text{het}}(t)$ function is dependent on the kinetics of precipitate formation and the physicochemical properties of the system (pH, temperature, presence of other components). When solving this system of equations, the experimental values of p_{het} were approximated by a theoretical equation (curve *5* in Fig. 8):

$$p_{het} = a + be^{-et}\ . \qquad (27)$$

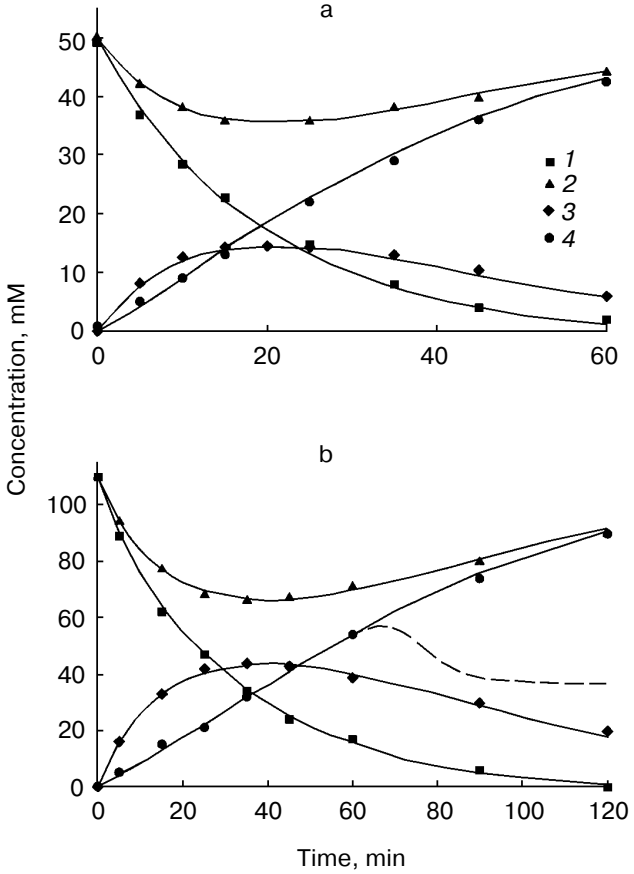


Fig. 7. Results of modeling of the enzymatic synthesis of ampicillin in homogeneous (a) and heterogeneous (b) systems. In the heterogeneous systems, a precipitate containing only D-PG is formed. Conditions: $E_0 = 3\cdot10^{-6}$ M, pH 6.3; 25°C. Designations: *1-4* refer to the concentrations of D-PGME, 6-APA, ampicillin, and D-PG, respectively. The dashed line corresponds to the concentration of D-PG in solution. The solid curves were computed by modeling.

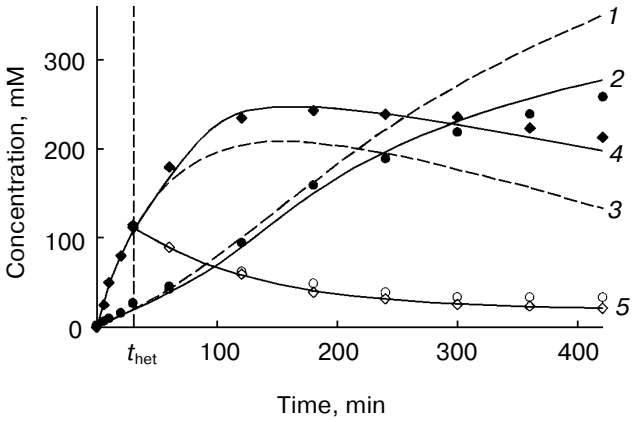


Fig. 8. Results of modeling the enzymatic synthesis of ampicillin in the heterogeneous system where both products of the reaction (D-PG and ampicillin) form a precipitate. The points indicate the experimental values of the total content of ampicillin (♦) and D-PG (•) and the concentrations of D-PG (○) and ampicillin (◇) in the solution. Results of modeling: *1*, *3*) kinetic curves of accumulation of D-PG and ampicillin calculated with Eqs. (14)-(18); *2*, *4*) kinetic curves calculated with Eqs. (22)-(26) having regard for the formation of precipitate of ampicillin (see text); *5*) time course of the change in the concentration of ampicillin in the solution calculated with Eq. (27). The vertical line shows the point in time at which the precipitate of ampicillin appears (t_{het}). Conditions: $s_0 = 0.5$ M, $n_0 = 0.28$ M, $e_0 = 2.3 \cdot 10^{-6}$ M, pH 6.3; 25°C.

When the precipitate of ampicillin is formed, we can use the solutions of two systems of equations to describe the enzymatic synthesis of ampicillin in the heterogeneous system. One system of equations (Eqs. (14)-(18)) describes the behavior of the reaction system to the point of appearance of ampicillin precipitate (t_{het} in Fig. 8). The other system of equations (Eqs. (22)-(26)), with initial conditions $t_0 = t_{het}$, $p = p_{het}$, $p_2 = p_{2het}$, $s = s_{het}$, and $n = n_{het}$ (concentrations of the components correspond to $t_0 = t_{het}$), describes the behavior of the reaction system containing the precipitate of ampicillin. This approach allowed us to get better agreement between the experimental data and the results of modeling (curves *2* and *4* in Fig. 8) and to explain the increase in the yield of the target product in such reaction systems in comparison with the homogeneous systems and the systems where only D-PG precipitate is formed. Results of calculation of the maximum yield of ampicillin obtained with this approach are given in Table 4. Thus, our mathematical method allows the progress curves for all the reaction components in the homogeneous and heterogeneous systems to be described to a good approximation and the effects of the kinetic parameters and the reaction conditions on the maximum yield of the target product to be analyzed. These results open opportunities for the elaboration of scientifically justified methods for optimization of enzymatic β-lactam antibiotic synthesis and allows the process to be *a priori* optimized on the basis of the experimental values of its kinetic parameters.

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