Enzymatic hydrolysis of β-lactam antibiotics at low pH in a two-phase "aqueous solution – water-immiscible organic solvent" system

Ghermes G. Chilov and Vytas K. Švedas

Abstract: The application of the two-phase "aqueous solution – water-immiscible organic solvent" system is suggested not for effective biocatalytic synthesis, but for hydrolytic purposes. Enzymatic hydrolysis of benzylpenicillin and *N*-phenylacetamidodesacetoxycephalosporanic acid to corresponding antibiotic nuclei 6-aminopenicillanic and 7-aminodesacetoxycephalosporanic acids in a two-phase water–butylacetate system at pH 3–4 is proposed as an alternative to the biocatalytic hydrolysis in an alkaline medium. An experimental study has been performed and a model has been developed, which describes the influence of pH, phase volume ratio, thermodynamic constants, and initial antibiotic concentration on the effectiveness of their hydrolysis in a two-phase "aqueous solution – water-immiscible organic solvent" system. The thermodynamic evaluation of penicillin G and 7-phenylacetamidodesacetoxycephalosporanic acid hydrolysis at low pH in a two-phase aqueous solution – water-immiscible organic solvent system has demonstrated high practical potential. The suggested approach allows for the exclusion of several technological steps during the transformation of natural β-lactam antibiotics to their semi-synthetic analogues: alkaline extraction of the biosynthetic antibiotic from butylacetate followed by its enzymatic hydrolysis at pH 7.5–8.0 and further acidification of the reaction mixture, which results in the precipitation of the antibiotic nucleus. Experimental observations also revealed a specific feature of this process: the kinetic supersaturation of the antibiotic nucleus slows down the attainment of the equilibrium, which should be taken into account when further developing this approach.

Key words: enzymatic hydrolysis, β-lactam antibiotic nuclei, two-phase systems, supersaturation, penicillin acylase.

Résumé : On propose l'utilisation du système à deux phases « solution aqueuse – solvant organique immiscible dans l'eau », non pas pour des synthèses biocatalytiques efficaces, mais pour des fins d'hydrolyse. On propose une hydrolyse enzymatique dans le système à deux phases eau–acétate de butyle à un pH allant de 3 à 4 comme alternative à l'hydrolyse biocatalytique en milieu alcalin de la benzylpénicilline et de l'acide *N*-phénylacétamidodésacétoxycéphalosporanique conduisant aux noyaux des antibiotiques correspondants, les acides 6-aminopénicillanique et 7-aminodésacétoxycéphalosporanique. On a effectué une étude expérimentale et on a développé un modèle qui permet de décrire l'influence du pH, du rapport des volumes dans la phase, des constantes thermodynamiques et de la concentration initiale d'antibiotique sur l'efficacité de leurs hydrolyses dans un système à deux phases « solution aqueuse – solvant organique à deux phases ». L'évaluation thermodynamique de l'hydrolyse de la pénicilline G et de l'acide 7-phénylacétamidodésacétoxycéphalosporanique à faible pH, dans un un système à deux phases « solution aqueuse – solvant organique à deux phases » a permis de démontrer le caractère très pratique de ce système. L'approche suggérée permet d'exclure plusieurs étapes technologiques de la transformation des antibiotiques β-lactames naturels en leurs analogues semi-synthétiques, telle l'extraction alcaline de l'antibiotique biosynthétique de l'acétate de butyle, suivie de son hydrolyse enzymatique à pH de 7,5 à 8,0 et de l'acidification subséquente du milieu réactionnel pour précipiter le noyau de l'antibiotique. Une réalisation expérimentale de cette approche a aussi permis de mettre en évidence une caractéristique spécifique du procédé: dans le développement de cette approche, on doit tenir compte du fait que la sursaturation cinétique du noyau antibiotique ralentit l'obtention de l'équilibre.

Mots clés : hydrolyse enzymatique, noyau antibiotique β-lactame, systèmes à deux phases, sursaturation, acylase de pénicilline.

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Dedicated to Professor J. Bryan Jones on the occasion of his 65th birthday.

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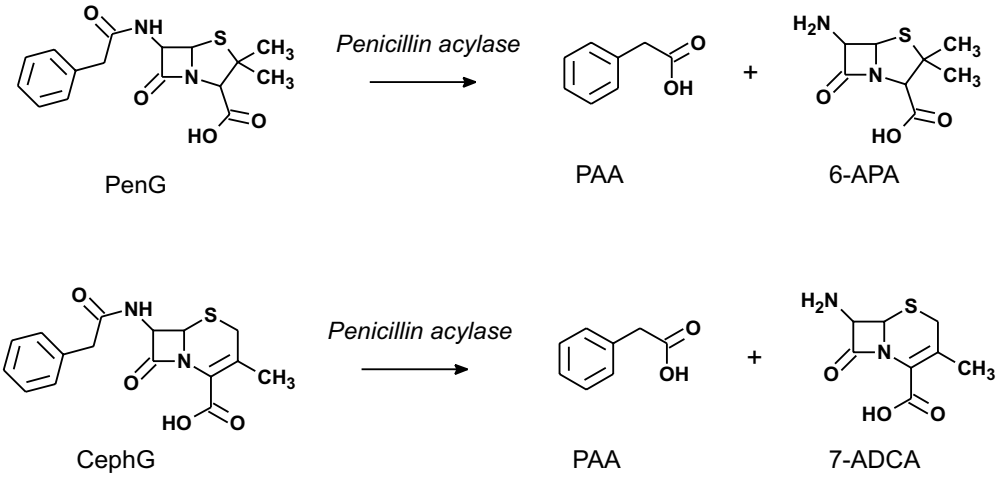
Fig. 1. Penicillin acylase catalyzed hydrolysis of benzylpenicillin (Pen G) and 7-phenylacetamidodesacetoxycephalosporanic acid (CephG) to antibiotic nuclei (6-APA and 7-ADCA, respectively) and phenylacetic acid (PAA).



Introduction

In a technological scheme of natural penicillin conversion to its semi-synthetic analogues, penicillin G (Pen G) is first extracted from the fermentation broth by organic solvent (butylacetate, amylacetate) at pH 2.0–2.5 (1). After back-extraction into aqueous solution, it is enzymatically hydrolyzed at pH 7–8 (Fig. 1). After acidification of the reaction mixture, the antibiotic nucleus is isolated by precipitation at its isoelectric point (pH 3.5) (2). Attractiveness of such process stems mainly from thermodynamic considerations, where antibiotic hydrolysis could reach nearly quantitative yields above pH 7 (3). Moreover, the optimum pH (7.5–8.0) of penicillin acylase catalytic activity coincides well with these conditions (4). The major shortcomings of the above-mentioned Pen G conversion to 6-aminopenicillanic acid (6-APA) include numerous pH shifts leading to formation of waste inorganic salts and degradation of a β-lactam ring in an alkaline medium. Additionally, kinetic factors such as strong product inhibition (competitive by phenylacetic acid and non-competitive by 6-APA) should be borne in mind (5). These drawbacks can, however, be avoided in the process presented in this work — the hydrolysis of biosynthetic β-lactam antibiotics Pen G and 7-phenylacetamidodesacetoxycephalosporanic acid (Ceph G) at pH 3–4 in a two-phase water–butylacetate system. Butylacetate containing the acidic form of the antibiotic can be directly added to an aqueous phase containing penicillin acylase, which is followed by partitioning of antibiotic between two phases, hydrolysis of the antibiotic in an aqueous phase, precipitation of an antibiotic nucleus (whose solubility in the considered pH range is the lowest possible), and partitioning of the second reaction product (phenylacetic acid) between the two phases. In addition to the reduction of technological steps, the suggested process can improve the quality and the yield of the reaction product since several factors leading to the degradation of β-lactam ring are excluded. It is worth noting that two-phase water-immiscible organic solvent systems have so far only been evaluated for performing synthetic reactions (6, 7), while the hydrolytic potential of these systems has been deprived of proper attention. For practical implementation of this process in hydrolysis (e.g., in the countercurrent biocatalytic reactor, as recently proposed by Straathof et al. (8)), it is necessary to conceive the thermodynamic and kinetic parameters of the system, which influence the effectiveness of this process. Here, a more detailed analysis of thermodynamic model of β-lactam antibiotic hydrolysis in a two-phase aqueous solution – water-immiscible organic solvent system is described and some kinetic features of the process are shown to be crucial. Critical evaluation of the experimental results and mathematic modeling demonstrate the potential of the proposed process.

Theoretical aspects

Thermodynamics of hydrolysis in a two-phase system at low pH

Thermodynamic regularities of antibiotic hydrolysis in a two-phase aqueous solution – water-immiscible organic solvent system should take into account the following physicochemical processes:

(*i*) Partition of acidic form of antibiotic (ABH) between two phases:

$$[1] \qquad ABH_{org} \Leftrightarrow ABH$$

with a partition constant of

$$[2] \qquad K_{P,ABH} = \frac{[ABH_{org}]}{[ABH]}$$

(*ii*) Dissociation of ABH in an aqueous phase (p*K* of the carboxylic group of β-lactam antibiotics is close to 2.5):

$$[3] \qquad ABH \Leftrightarrow AB^- + H^+$$

with a dissociation constant of

$$[4] \qquad K_{a,ABH} = \frac{[H^+][AB^-]}{[ABH]}$$

(*iii*) Enzymatic hydrolysis in an aqueous phase:

$$[5] \qquad AB^- + H_2O \Leftrightarrow AH + B^-$$

and the corresponding equilibrium constant:

$$[6] \quad K_{synth,AB} = \frac{[AB^-]}{[AH][B^-]}$$

where AH is a protonated form of phenylacetic acid and B^- is an anionic form of antibiotic nucleus.

(*iv*) Partition of a protonated form of phenylacetic acid between two phases:

$$[7] \quad AH \Leftrightarrow AH_{org}$$

with a partition constant of

$$[8] \quad K_{P,AH} = \frac{[AH_{org}]}{[AH]}$$

(*v*) Dissociation of phenylacetic acid in an aqueous phase:

$$[9] \quad AH \Leftrightarrow A^- + H^+$$

with a dissociation constant (p*K* 4.3) of

$$[10] \quad K_{a,AH} = \frac{[H^+][A^-]}{[AH]}$$

(*vi*) Protonation of an amino group of the antibiotic nucleus (p*K* about 4.7):

$$[11] \quad B^- + H^+ \Leftrightarrow BH^{\pm}$$

with a dissociation constant of

$$[12] \quad K_{a2,BH} = \frac{[B^-][H^+]}{[BH^{\pm}]}$$

(*vii*) Protonation of a carboxylic group of the nucleus (p*K* 2.5):

$$[13] \quad BH^{\pm} + H^+ \Leftrightarrow BH_2^{\pm}$$

with a dissociation constant of the Zwitter-ionic form of

$$[14] \quad K_{a1,BH} = \frac{[BH^{\pm}][H^+]}{[BH_2^+]}$$

(*viii*) Precipitation of the Zwitter-ionic form of antibiotic nucleus:

$$[15] \quad BH^{\pm} \Leftrightarrow BH^+_{ppt}$$

which should make its concentration in an aqueous phase equal to its solubility:

$$[16] \quad [BH^{\pm}] = [BH^{\pm}_{sol}]$$

Bearing in mind the relations mentioned above, we can trace the factors that determine the yield of antibiotic nucleus in the corresponding hydrolysis. Reserving η for the reaction yield, the total content of each component (i.e., concentrations of all ionic forms in the co-existing phases) can be represented as

$$[17] \quad [AB] = (1 - \eta)[AB]_0$$

$$[18] \quad [A] = \eta[AB]_0$$

$$[19] \quad [B] = \eta[AB]_0$$

Concentration of a certain ionic species in an aqueous medium can be expressed as

$$[20] \quad [AB^-] = [AB]F_{AB^-}$$

$$[21] \quad [AH] = [A]F_{AH}$$

where

$$[22] \quad F_{AB} = \frac{1}{1 + \frac{[H^+]}{K_{a,ABH}}(1 + \alpha K_{P,ABH})}$$

$$[23] \quad F_{AH} = \frac{1}{1 + \frac{[H^+]}{K_{a,AH}}(1 + \alpha K_{P,AH})}$$

which follow from the eqs. [2], [4], [8], and [10], respectively (where α is the phase-volume ratio). If concentration of the Zwitter-ionic form is confined by its solubility $[BH^{\pm}_{sol}]$,

$$[24] \quad [B] = [BH^{\pm}_{sol}]\frac{K_{a1,B}}{[H^+]}$$

then the thermodynamic equilibrium of hydrolysis can be expressed in the following way:

$$[25] \quad K_{synth,AB} = \frac{(1-\eta)[AB]_0 F_{AB}}{\eta[AB]_0 F_{AH}[BH^{\pm}_{sol}]\frac{K_{a1,B}}{[H^+]}}$$

The final expression for the reaction yield can be written as

$$[26] \quad \eta = \frac{1}{1 + K_{synth,AB}[BH^{\pm}_{sol}]\frac{K_{a1,B}}{[H^+]}\frac{F_{AH}}{F_{AB}}}$$

Factors that influence the yield of hydrolysis

As seen from the previous paragraph, the yield of the antibiotic nucleus is a complex parameter determined by the thermodynamic constant of hydrolysis, the dissociation and partition constants, and the solubility of the product. Using eq. [26], we can estimate their impact on the outcome of the process.

The effectiveness of the hydrolysis in a two-phase system under a given set of conditions does not depend on the initial antibiotic concentration. Such a conclusion might seem strange, especially if one takes into consideration that in the industrial processing of penicillin, hydrolysis at pH 7–8 (its starting concentration) does not exceed 0.25 M — because of the thermodynamic limitations. A further increase of the substrate concentration would lead to the decrease in the yield of 6-APA (9). But this conclusion is only valid for homogeneous solutions. If precipitation of the product(s) occurs, such a dependence on the initial substrate concentration would also disappear. From a practical point of view, the larger the substrate concentration converted, the higher the productivity of the process.

In evaluating the influence of different factors on the degree of antibiotic conversion, it is important to note that when some characteristics of the two-phase system (thermo-

dynamic constant of hydrolysis, dissociation and partition constants of certain reaction components) are fixed, parameters such as pH and phase volume ratio (α) represent extra degrees of freedom, which must be taken into account when looking for optimal processing conditions.

The pH dependence of a conversion is stipulated by the presence of ionogenic groups capable of dissociation in a pH range of 3–4. It follows from thermodynamic considerations that hydrolysis in an alkaline medium is nearly irreversible. Degradation of β-lactam rings at higher pH, however, limits its application for the biocatalytic process in this pH range, making the biocatalytic process optimal in a pH range of 7–8. In a two-phase system, the situation is quite different. Removal of both reaction products out of the reaction sphere (i.e., precipitation of 6-APA and partition of phenylacetic acid) promotes enzymatic hydrolysis, making the biocatalytic process optimal in acidic medium. In a two-phase system, a pH increase above pH 4.4 hampers extraction of phenylacetic acid into the organic phase, prevents 6-APA precipitation, and, in contrast to homogeneous reactions, does not improve hydrolysis. Analysis of eq. [26] shows that with a pH decrease, antibiotic conversion approaches a definite value:

$$[27]\quad \eta_{\mathrm{pH}\to 0} = \frac{1}{1 + K_{synth,\mathrm{AB}}\,[\mathrm{BH}^{\pm}_{\mathrm{sol}}]\dfrac{K_{a1,\mathrm{B}}}{K_{a,\mathrm{AB}}}\dfrac{1 + K_{P,\mathrm{ABH}}\,\alpha}{1 + K_{P,\mathrm{AH}}\,\alpha}}$$

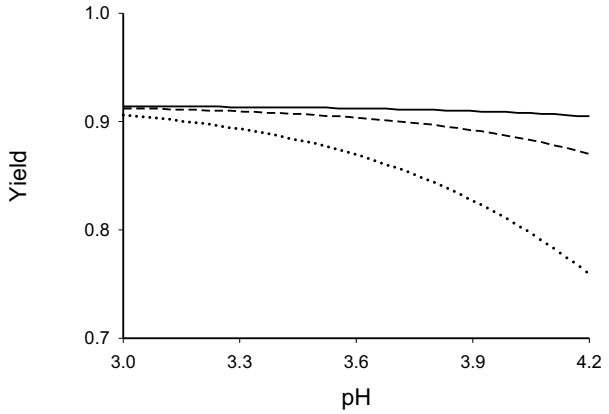
It is quite clear that the optimal yield is determined both by thermodynamic characteristics and phase volume ratio (α). The influence of the latter parameter, however, is quite limited. Generally, the values of the partition constants are several tens in its magnitude, and hence under reasonable values of α the value of $K_P\alpha$ sufficiently exceeds the unity. Taking this into consideration, eq. [27] may be reduced to

$$[28]\quad \eta_{\mathrm{pH}\to 0} = \frac{1}{1 + K_{synth,\mathrm{AB}}\,[\mathrm{BH}^{\pm}_{\mathrm{sol}}]\dfrac{K_{a1,\mathrm{B}}}{K_{a,\mathrm{AB}}}\dfrac{K_{P,\mathrm{ABH}}}{K_{P,\mathrm{AH}}}}$$

Finally, within the set of thermodynamic parameters and their combinations (which determine the yield), we can distinguish three basic parameters: (*i*) The product of the thermodynamic antibiotic synthesis constant and the solubility of the Zwitter-ionic form of the nucleus ($K_{synth,\mathrm{AB}}[\mathrm{BH}^{\pm}_{\mathrm{sol}}]$). It should be emphasized that the impact of these two parameters on the yield is reciprocal, i.e., any deterioration of the thermodynamics of the hydrolysis could, in principle, be compensated for by lowering the product solubility. (*ii*) The difference in the p*K* of the amino group of antibiotic nuclei and the carboxylic group of the substrate, where protonation of the former leads to the domination of the Zwitter-ionic form (low solubility), while deprotonation of the latter assists in the extraction of the substrate into aqueous phase. (*iii*) The ratio of the partition constants of antibiotic and the phenylacetic acid, where partition of the substrate into the aqueous phase and product withdrawal to the organic phase favors conversion.

Of particular interest is determining the degree of conversion under given conditions. This is especially intriguing, since biocatalytic hydrolysis at pH 7.5–8 was already shown to be quite acceptable. To illustrate this point, we first analyzed the dependence of the yield of 6-APA, calculated on the basis of the model presented above (Fig. 2), using thermodynamic parameters from the literature (Table 1).

Fig. 2. Dependence of 6-APA yield during Pen G hydrolysis in a two-phase system, calculated with the eq. [18] on the basis of constants given in Table 1. Dashed line $\alpha = 1$; solid line $\alpha = 5$; dotted line $\alpha = 0.2$.



According to thermodynamic predictions, the yield of 6-APA under pH 3–4 with a single hydrolytic step is close to 90%, which may be considered quite satisfactory. But one should determine whether such yields are achievable in practice and if the kinetics of the thermodynamically controlled hydrolysis of antibiotics under a given set of conditions is achievable. The aim of this paper is to report the results of Pen G and Ceph G hydrolysis in a two-phase aqueous solution – water-immiscible organic solvent system at acidic pH.

Materials and methods

Materials

6-APA, 7-ADCA, Pen G-K salt, Ceph G, and penicillin acylase from *E. coli* (ATCC 11105) were kind gifts from DSM (the Netherlands). The concentration of the enzyme-active sites was determined by titration with PMSF (15) and found to be 3.2×10^{-4} M. PAA was from Aldrich, butyl acetate from Fluka, acetonitrile (HPLC grade) from Kriochrom (Russia), sodium dodecylsulfate, the components of the buffer systems, and all other reagents were from Merck. In all experiments MilliQ (Millipore) water was used.

Enzymatic hydrolysis in a two-phase system

Each experiment was performed in a series of microtubes (Eppendorf) in a shaker at 20°C. The total reaction volume was 500 μL. A stock solution of Pen G (0.1 M) was prepared by dissolving the corresponding amount of Pen G–K salt in water. Equal volumes of BuAc and Pen G stock solutions (250 μL of each) were placed in a microtube and the pH was adjusted to a required value with phosphoric acid (less than 5 μL). At cephalosporin hydrolysis, a definite amount of Ceph G (acidic form) was placed in 250 μL of BuAc, the suspension was then treated in an ultrasonic bath (Branson 2510) for 10 min to dissolve the substrate, the required volume of water was added, the system was stirred in the centrifuge, and the pH was adjusted with a KOH solution.

Table 1. Thermodynamic parameters of the processes involved in antibiotic hydrolysis in a two-phase system under acidic pH.

	Pen G	Ceph G	PAA	6-APA	7-ADCA
K_{synth} (M^{-1})	680[a]	n.d.	—	—	—
pK_{COOH}	2.5[b]	2.5[b]	4.3[b]	2.6[c]	2.2[d]
pK_{NH2}	—	—	—	4.7[c]	4.9[d]
K_P	47[e]	n.d.	28[e]	0	0
Solubility (M)	—	—	—	0.01[c]	0.001[d]

[a]Values from ref. 10.
[b]Values from ref. 11.
[c]Values from ref. 12.
[d]Values from ref. 13.
[e]Values from ref. 14.

Reactions were started by adding 7.8 μL of stock PA solution.

Reactions in close-to-equilibrium systems

Reaction conditions (e.g., total reaction volume, temperature, phase volume ratio) were similar to those described above. Stock solutions were prepared by dissolving definite amounts of antibiotic and phenylacetic acid in water; after mixing with BuAc, a required amount of solid 6-APA (or 7-ADCA) was added, and the pH was adjusted with phosphoric acid.

Analysis

To analyze the total content of both the aqueous and organic phases, 10 mL of the stop-reagent (50% of HPLC eluent (v/v) and acetonitrile) was added to the reaction mixture (500 μL) at a definite moment. An aliquot of the obtained solution was diluted with the eluent [potassium phosphate buffer (0.68 g L^{-1}, pH 3.0), sodium dodecylsulfate (100 mg L^{-1}), and CH_3CN (24%, v/v)] and analyzed by HPLC using a Waters system with a Chrompack Nucleosil-C18 4.6 × 250 mm column, Waters Lambda-Max Model 481 detector at 210 nm, and a Waters 6000A pump with a flow of 1 mL min^{-1}. Once the 6-APA concentration in an aqueous phase was determined, the reaction mixture was centrifuged for 1 min at 13 200 rpm (Eppendorf 5415D), and then 10 μL of the aqueous phase were added to 1 mL of the stop-reagent. After dilution with the eluent, an aliquot was subjected to HPLC analysis.

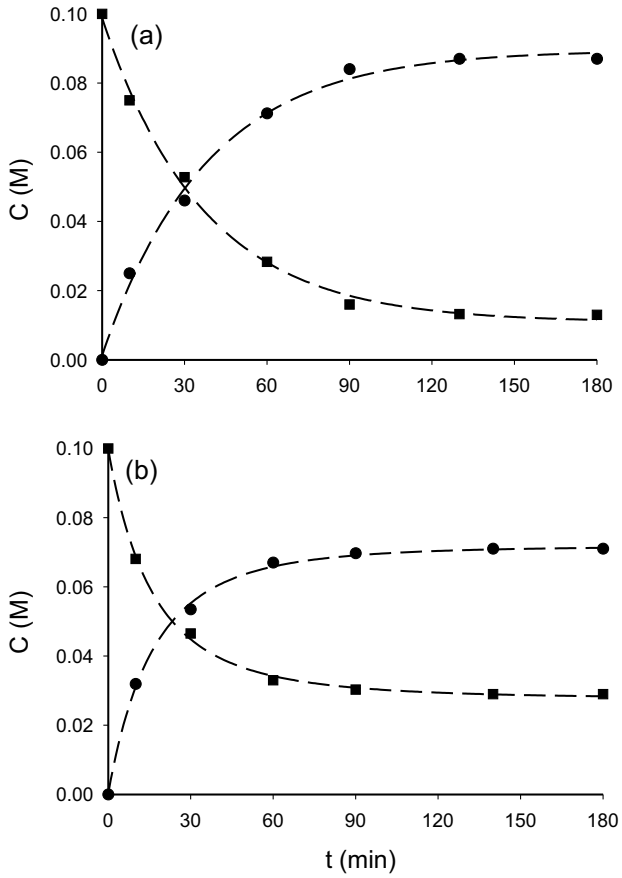
Results and discussion

Hydrolysis of antibiotics

Antibiotic hydrolysis in a two-phase aqueous solution – water-immiscible organic solvent system could be complicated by several factors, with the most negative ones being enzyme inactivation in the organic phase at low pH and β-lactam degradation.

The presence of a water-immiscible organic phase did not influence the activity or stability of native penicillin acylase: the loss of activity during the characteristic time of reaction (1–3 h) did not exceed 10%, which is quite acceptable for the thermodynamic evaluation of the system. Stabilized enzyme preparations should therefore be used in a preparative biocatalytic process to meet the economic and technological demands. In this study, we focused primarily on a thermodynamic evaluation of the new approach, and hence, did not apply standard techniques for PA stabilization (16, 17).

Fig. 3. Integral curves of Pen G (*a*) and Ceph G (*b*) hydrolysis in a two-phase system. (*a*) pH 3.8, 10 μM PA; (*b*) pH 3.5, 10 μM PA; (■), antibiotic (●), antibiotic nucleus.

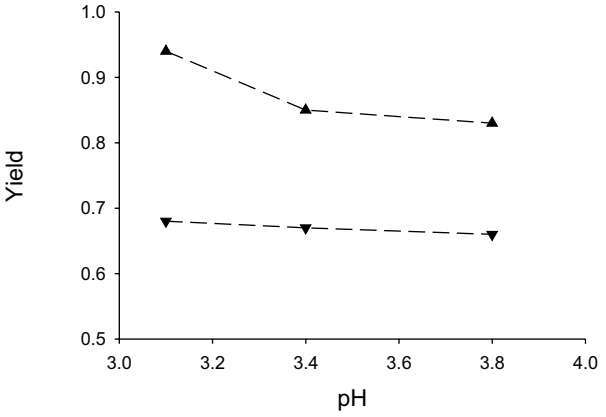


Penicillin degradation was studied by Reschke and Schugerl (1), who observed lower stability of penicillin in an aqueous phase under pH 3–4 and no degradation in the organic phase. Based on their experimental data and accounting for the partition of antibiotic in a two-phase system (Table 1), we determined the chance of penicillin degradation at the stated experimental conditions to be less than 1%. In fact, we did not observe Pen G decomposition within an hour in a two-phase system at pH 3.5. Since Ceph G is known to be more stable in an acidic medium, there was no danger of decomposition.

Typical experimental integral curves of penicillin acylase-catalyzed β-lactam antibiotic hydrolysis in a two-phase system are given in Fig. 3. Effective enzymatic hydrolysis of both biosynthetic antibiotics Pen G and Ceph G took place at pH 3.8, followed by precipitation of corresponding antibiotic nuclei, which is in good agreement with the preliminary thermodynamic evaluation.

Withdrawal of both reaction products out of reaction sphere, i.e., precipitation of 6-APA (or 7-ADCA) and extraction of phenylacetic acid into the organic phase, is favorable from both a thermodynamic and a kinetic point of view. The pH dynamics during the course of hydrolysis must, however, being taken into consideration. Starting from the initial pH (3–4), there was no significant change in pH over the course

Fig. 4. Experimental dependence of conversion degrees in hydrolysis of 0.1 M antibiotic in a two-phase system. (▼), 6-APA; (▲), 7-ADCA.



of the reaction due to the balance of the dissociation and partition constants, which prevented formation of unwanted inorganic salts in a reaction mixture.

Experimental antibiotic nuclei yield dependence on pH

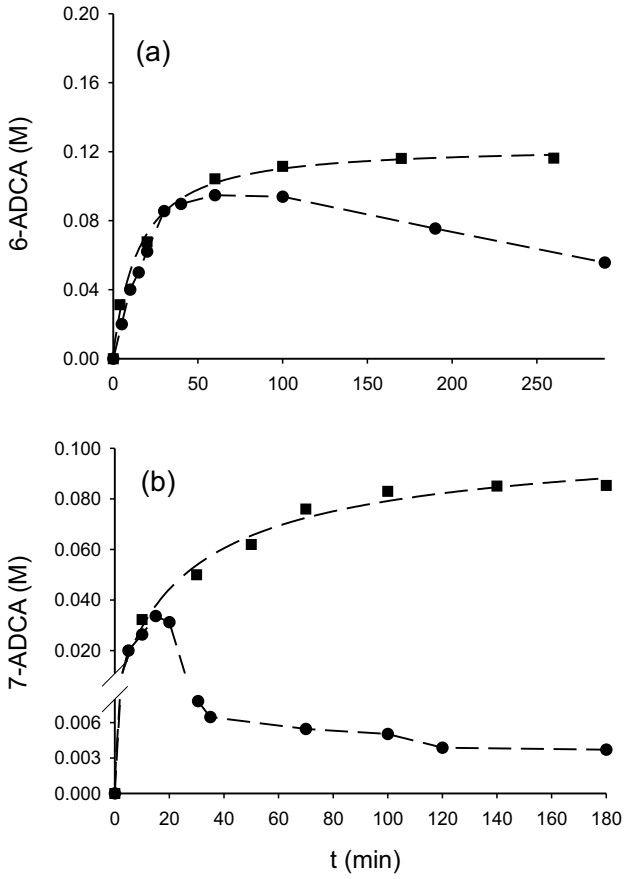
Hydrolysis of Pen G and Ceph G in a two-phase system (water–BuAc, 1:1) at pH 3–4 was carried out and the pH dependencies of the antibiotic conversion were obtained (Fig. 4).

In a chosen interval, the yield of hydrolysis (70% for 6-APA and 90% for 7-ADCA) was weakly dependent on pH. It should be noted that the yield of 6-APA evaluated on the basis of reported parameters (Fig. 2) exceeds the experimental yields by 20%. The discrepancy could arise from slightly different experimental conditions used in different laboratories when determining the numerous constants used for the evaluation (eq. [27] contains several parameters determined by different scientists). For example, there is a remarkable discrepancy for the Pen G synthesis constant: Švedas et al. (3) reported a value of 4300 M^{-1}, while Tewari and Goldberg (10) determined the value to be 680 M^{-1}. Bearing this in mind, we tested the thermodynamic model presented above, but initially sought to characterize the equilibrium state during the course of antibiotic hydrolysis in a two-phase system.

It is interesting to compare antibiotic hydrolysis under studied conditions in a biphasic system with that in a monophasic one at the same acidic pH and at traditionally used alkaline conditions (pH 8). Pen G and Ceph G hydrolysis in a monophasic aqueous system at low pH is thermodynamically unfavorable (see corresponding values of equilibrium constants) and is not practical due to very low product yield (less than 30%) as well as low conversion rates due mainly to very strong product inhibition at these conditions (data not shown). Thus, in comparison with the corresponding monophasic aqueous system, the biphasic system (where both reaction products are removed out of the reaction sphere due to extraction of phenylacetic acid into the organic phase and precipitation of 6-APA or 7-ADCA) favors the hydrolytic reaction with thermodynamic and kinetic feasibility.

Compared with the traditional hydrolysis of Pen G and Ceph G at pH 8, the suggested approach is subjected to less

Fig. 5. Supersaturation at antibiotic nucleus formation in the course of Pen G (*a*) and Ceph G (*b*) hydrolysis. (■), total antibiotic nucleus content; (●), concentration in aqueous phase; (……), saturation level.



product inhibition, and therefore is just 5 to 6 times slower. The total turnover rate is good enough for preparative conversion. Moreover, the conversion rate can be further increased by the appropriate choice of "acidic" PA, since enzymes of this family are remarkably different in their pH dependencies of catalytic activity (18). Furthermore, the overall antibiotic conversion in a suggested technological process can be increased up to the stoichiometric value by applying a continuous downstream processing scheme. Antibiotic extraction from a cultural broth can be coupled with further conversion in the biphasic system, which allows for downstreaming in the feed-countercurrent reactor, and thus, the possibility of reaching conversions that are close to unity (19). When comparing traditional hydrolysis with a putative one, the overall balance should take into account factors such as the decrease in the number of the technological steps, the formation of waste inorganic salt during numerous pH shifts in the traditional procedure, and the economic gains. It should also be done on the experimental process at the preparative scale.

Dependence of the antibiotic conversion on its initial concentration

The basic qualitative feature of the thermodynamic model of β-lactam antibiotic hydrolysis in a two-phase system with precipitation of the antibiotic nucleus is the independence of

Fig. 6. Reaction in a two-phase system under close-to-equilibrium conditions. (*a*) (▲), 87 mM 6-APA, 87 mM PAA, 13 mM Pen G; (■), 89 mM 6-APA, 89 mM PAA, 11 mM Pen G; (●), 95 mM 6-APA, 95 mM PAA, 5 mM Pen G; (*b*) (▲), 98 mM 7-ADCA, 98 mM PAA, 2 mM Ceph G; (■), 99 mM 7-ADCA, 99 mM PAA, 1 mM Ceph G; (●), 100 mM 7-ADCA, 100 mM PAA, 0 mM Ceph G.

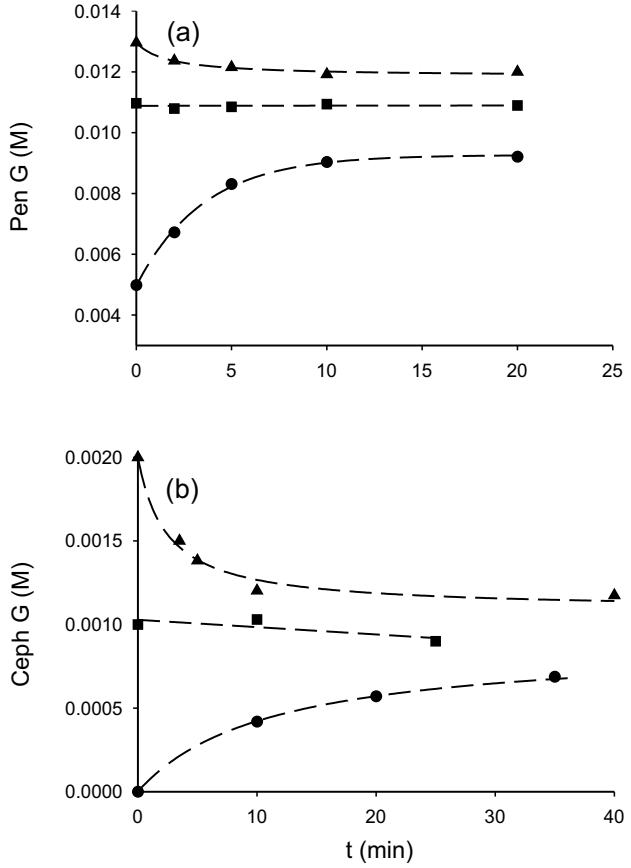
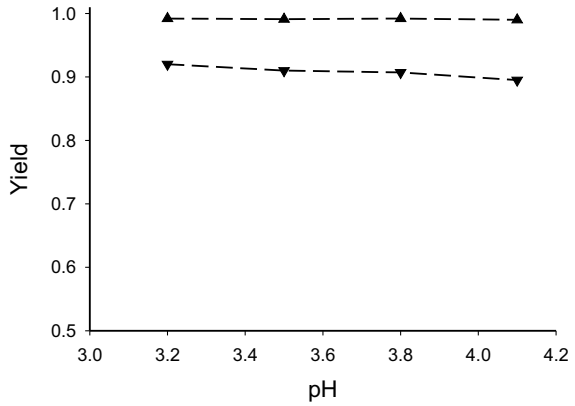


Fig. 7. Dependence of thermodynamically controlled antibiotic conversion degree in a two-phase system. (▼), 6-APA; (▲), 7-ADCA.



the conversion on the initial substrate concentration. Experimental study shows that this is a case for Ceph G hydrolysis, where the yield of 7-ADCA does not depend on the Ceph G concentration up to the 0.1 M (solubility limit of Ceph G under these conditions). An increase in the Pen G concentration from 0.1 to 0.2 M, however, decreases the yield of 6-APA from 67 to 57%. This fact pointed out one more discrepancy between the thermodynamic model and the experimental data. To solve this apparent contradiction, one might change the model, but there was no sufficient reason for such a change, and we wanted to be sure that the experimental data reflected a true equilibrium state. (Most of the doubts were related to the precipitation of 6-APA.)

Dynamic supersaturation of 6-APA in the course of reaction

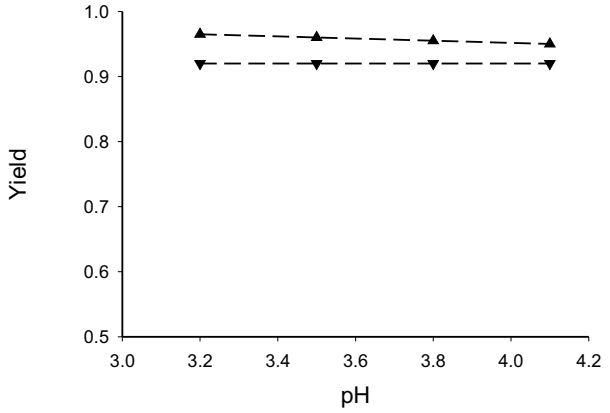
Investigation of supersaturation effects was carried out by comparing the composition of aqueous phase with that of the whole system. Results are presented in Fig. 5.

Supersaturation takes place in both cases, and its scale is impressive: 12-fold for 6-APA and 35-fold for 7-ADCA. It therefore makes sense to consider these results in more detail. Normally, supersaturated solutions are usually unstable and supersaturation dissipates within several minutes, but this is not the case in this particular system. The process of antibiotic nuclei crystallization is complicated by various factors such as contact with the organic phase, presence of other reagents (Pen G and PAA), and the enzyme. In a separate experiment, we observed that addition of 6-APA or 7-ADCA crystals into the corresponding reaction mixture does not influence precipitation, which emphasizes the peculiarity of solid-phase formation under these experimental conditions. We noted that 6-APA supersaturation dissipates very slowly (tens of hours) and within the reasonable time interval, one cannot expect the concentration of antibiotic nuclei in aqueous phase to reach equilibrium. Despite a more sharp precipitation for 7-ADCA, equilibrium solubility is also not reached, and fourfold of oversaturation is left. Thus the stability of supersaturated solutions of antibiotic nuclei is most probably responsible for the discrepancy between the experimental and thermodynamic modeling presented above.

True equilibrium state of hydrolysis in a two-phase system

It is nevertheless important to know what yields could be expected if the problems with precipitation of the nuclei were solved. In other words, it is important to determine the true equilibrium state of hydrolysis in a two-phase system. For this purpose, we investigated the direction of the reaction in systems with designed composition of reagents (Pen G, 6-APA, PAA). By tracing the reaction direction it can be concluded where the equilibrium, the state of no macroscopic reagent conversion, is situated. At the same time there is no need to wait for the end of the reaction — it is enough just to check the initial direction and then again with each iteration step as the reaction approaches those concentrations that characterize the system at equilibrium. An analogous method is well known in mathematics as the method of division by half for root estimation, where the sign of a function is checked at each iteration step. If the sign changes, the size of the interval, which contains the root or the equilibrium (as in our case), is reduced. This method is even more attractive because only the initial interval of reaction is taken into account and thus supersaturation, reagent degradation, and other dynamic effects can be omitted.

Fig. 8. Expected degrees of conversion in a two-phase system after formal elimination of supersaturation in accordance with eq. [21]. (▼), 6-APA; (▲), 7-ADCA.



Several typical dependencies are given in Fig. 6 to illustrate this proposed approach.

As can be seen, loading the system with 0.087 M 6-APA, 0.087 M PAA, and 0.013 M Pen G (which corresponds to 87% yield) leads to further hydrolysis of the antibiotic, affording equilibrium yields exceeding 87%. On the other hand, designing a yield of 95% causes a synthetic reaction, and thus the equilibrium value is less than 95%. Finally, in the system containing 0.089 M 6-APA, 0.089 M PAA, and 0.011 M Pen G (89% yield), no macroscopic changes in the concentrations were observed, which suggests a good approach to achieving equilibrium conditions.

A series of measurements under pH 3.2–4.1 was conducted and the thermodynamic equilibrium state of Pen G and Ceph G hydrolysis in a two-phase system (water–BuAc) was found. Results are presented in Fig. 7.

Correspondence of true equilibrium state and practical conversions

In the case of Pen G, thermodynamically estimated yields of hydrolysis (Fig. 2) are in good agreement with experimental values obtained under close-to-equilibrium conditions (Fig. 7). Moreover, the latter values also agreed with the yields in non-equilibrium (as far as nucleus precipitation is concerned) reactions. In fact, if the crystallization process is considered relatively slow in comparison with the chemical reaction, the yield will be determined by eq. [26], where the equilibrium solubility should be replaced by the current concentration (determined by supersaturation) in the aqueous phase. Then, other thermodynamic parameters being equal, the difference between thermodynamically and kinetically controlled yields will be stipulated only by the ratio of saturated and current concentrations according to eq. [26]. After several transformations, we obtain the following parametric relation between the conversion degrees and the ratio of concentrations of precipitating component:

$$[29]\quad \frac{[BH^{\pm}_{eq}]}{[BH^{\pm}_{kin}]} = \frac{\frac{1}{\eta_{eq}} - 1}{\frac{1}{\eta_{kin}} - 1}$$

where $[BH^{\pm}_{eq}]$ is the saturated (equilibrium) level and $[BH^{\pm}_{kin}]$ is the supersaturated (kinetically stipulated) concentration; η_{eq} and η_{kin} are the respective yields. Thus, on the basis of supersaturations observed, one can recalculate expected yields with the use of eq. [29]. Results of these calculations are presented in Fig. 8.

As can be seen, after formal elimination of supersaturation, the expected yields approximate the equilibrium values with good accuracy. This fact highlights that both the experimental results and the theoretical conceptions intrinsically agree.

Conclusion

A process of Pen G and Ceph G hydrolysis is studied in a two-phase system (water–BuAc) at pH 3–4. The thermodynamic model of the process is analyzed, incorporating the influence of pH, phase volume ratio, initial substrate concentration, and parameters of hydrolytic reaction (acid-base and interphase equilibria). For effective hydrolysis, we emphasize the crucial role of product withdrawal from the reaction sphere in a two-phase system: extraction of the emerging phenylacetic acid as well as precipitation of the antibiotic nucleus during the course of hydrolysis result in high yields. Experimental tests also revealed the formation of antibiotic-nucleus supersaturated solutions. For these solutions, being rather stable, the yield in hydrolytic reactions is smaller than predicted by the thermodynamic model. To study the true thermodynamic equilibrium state, a method is proposed where the initial reagent concentrations in an experimental system are set close to the equilibrium concentration values. Depending on whether hydrolysis or synthesis takes place, the interval of equilibrium concentrations is returned. Supersaturation effect is shown to decrease 6-APA yield from 90%, as it is set by thermodynamics, to 67%, and 7-ADCA yield from 99 to 90%, correspondingly.

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List of symbols

α	Phase volume ratio (V_{org}/V_{aq})
$C(X)$	Cconcentration of species X (mol L^{-1})
$C_0(X)$	Initial concentration of X (mol L^{-1})
$F(X)$	Mol fraction of species X
K_a	Acidity dissociation constant (mol L^{-1})
pK	Negative logarithm of acidity dissociation constant
K_P	Partitioning constant
K_{synth}	Equilibrium synthesis constant (mol^{-1} L)
V	Volume (L)
η	Yield

Sub- or superscript

app	Apparent
aq	Aqueous
eq	Equilibrium
kin	Kinetic
org	Organic
sol	Solubility
ppt	Precipitate