0273-2289/91/2703—0277$2.80

Equilibrium Controlled Synthesis of Cephalothin in Water-Cosolvent Systems by Stabilized Penicillin G Acylase

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Received July 21, 1989; Accepted May 15, 1990

ABSTRACT

Synthesis of cephalothin from thienylacetic acid (TAA) and 7-aminocephalosporanic acid (7ACA) has been carried out in the presence of high concentrations of organic cosolvents (e.g., 50% N,*N*'dimethylformamide) and under a wide range of experimental conditions (pH, temperature, etc.) by using very active and highly stabilized derivatives of Penicillin G acylase. We have been able to find the compromising solutions under which: (a) synthetic yields were markedly increased compared to those obtained in fully aqueous medium, (b) derivatives preserved a good percentage of catalytic activity, (c) derivatives were quite stable, and (d) high concentrations of substrates could be used. Under optimal conditions, 50 m*M* solutions of 7ACA in the presence of a slight excess of TAA were converted to cephalothin with yields higher than 95% and final concentrations of product up to 20 g/L were obtained.

Index Entries: Enzymes in organic synthesis; enzymes in organic solvents; cephalothin synthesis; penicillin G acylase.

INTRODUCTION

Enzymatic synthesis of semisynthetic antibiotics catalyzed by penicillin G acylase has interesting industrial perspectives: Protection of the carboxyl group of "antibiotic nucleus" becomes unnecessary because of enzyme

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specificity and there is not the risk of racemization when the acyl donor is a chiral compound (e.g., D-phenylglicine).

From a theoretical point of view, equilibrium controlled synthesis of semisynthetic antibiotics in monophasic water-organic cosolvent mixtures seems to be the easiest synthetic strategy. This is because: (a) activation of the acyl donor becomes unnecessary, (b) differences between pKs of carboxylic and amine groups are small (e.g., only 0.65 for cephalothin synthesis (*1*)) and therefore only moderately high concentrations of organic cosolvents are necessary to get very high synthetic yields, (c) synthetic yield corresponding to the most expensive component (in this case the nucleophile) can be increased, near to 100%, by using an excess of the less expensive component and (d) subsequent purification step may be quite simple (we only need the separation of the synthetic product from the excess of one component).

The main drawback of this thermodynamically controlled synthetic strategy is the deleterious effect that the presence of moderate to high concentrations of organic cosolvent (which are required to displace the equilibrium of synthesis) may exert on the activity and stability of the enzyme. As a result of this problem, only a few papers have been published concerning this equilibrium synthetic approach (*1–3*). On the contrary, a more complex strategy, the kinetically controlled synthesis in fully aqueous media, is quite popular in literature (*4*) in spite of its many practical disadvantages; the need for activated acyl donors, the transient nature of synthetic yields, the need for a large excess of nucleophile (often, the more expensive and instable component). In our opinion, the widespread acceptance of this strategy has its origin in the stability of the enzyme in aqueous media in spite of its lower practical interest.

We have developed an integrated strategy for the stabilization of penicillin G acylase by multipoint covalent attachment enzyme (amine)–support (aldehyde) of two different acylases from *E. coli* and from *K. citrophila*. In this way, we have been able to prepare acylase derivatives with very high thermal stabilization (up to 10,000-fold more stable than soluble or one-point attached immobilized penicillin G acylase from *E. coli*, these latter type of derivatives being the most currently used in the industry for hydrolysis of penicillin G catalyzed by this enzyme) (*5,6*). Since thermal stabilization parallels with stabilization in the presence of organic cosolvents (*7*), these derivatives seem to present important perspectives to be used as catalysts of the synthetic reactions previously pointed out.

We have previously investigated the equilibrium controlled synthesis of penicillin G, from phenylacetic acid and 6-aminopenicillanic acid, as a model reaction. We have tested the integrated effect of factors defining the water-cosolvent monophasic system (nature and concentration of the organic cosolvent, pH, temperature) on activity, stability, synthetic yields, substrate, and product solubility (*8,9*). As a compromise solution (keeping in mind all these important practical requirements) we have been able

to establish the "optimal conditions" for this model synthetic reaction: 50% N,*N*'dimethylformamide–water mixtures, at pH 6.5 and 4°C.

In this paper, we present a study of the equilibrium controlled synthesis of a very interesting antibiotic, cephalothin, from thienyl acetic acid and 7-aminocephalosporanic acid. First, we present a study of the stability of different PGA-agarose derivatives in experimental conditions similar to the ones to be used for our synthetic approach. From this study, we chose the most suitable derivative to study the cephalothin synthesis. Further, we experimentally tested whether the thermodynamics of cephalothin synthesis were similar to that of our model synthetic reaction. In addition, we studied the reaction rates of cephalothin synthesis as compared with the ones of penicillin G synthesis and finally, we tested the solubility of TAA and 7ACA and developed a "dynamic synthetic strategy," which allowed us to obtain very high synthetic yields by using very high substrate concentrations.

MATERIALS AND METHODS

Materials

Semipurified extracts of penicillin G acylases from *E.coli* and from *K.citrophila* were generously donated by Boehringer Mannheim GmbH (Penzberg, FDR) and by Antibioticos S.A. (León, Spain), respectively. 7-Aminocephalosporanic acid was a generous gift from CIPAN S.A.R.L. (Lisbon, Portugal). Sepharose CL 6B was purchased from Pharmacia Fine Chemicals (Uppsala, Sweden), thienyl acetic acid from Aldrich-Chemie, and all other reagents and substrates were from Sigma Chemical Co. (St. Louis, MO).

Preparation of PGA (amine)–Sepharose CL 6B (aldehyde) Derivatives

Activation of agarose gels (preparation of glyoxyl-agarose, Ag-O-CH_2-CHO) and further control of the PGA (amine)-agarose (aldehyde) multiinteraction process was performed as previously reported for trypsin (*10,11*). We have prepared a number of PGA derivatives having different activity/stability binomials (*5*), the main properties of which are listed in Table 1.

Enzymatic Assays

Hydrolytic Activity

In some cases (e.g., experiments of irreversible inactivation in organic solvents), we measured hydrolytic activity of our derivatives. This assay was performed by using penicillin G as a substrate. We titrated the liber-

Table 1
Main Properties of Penicillin G acylase-agarose derivatives

Derivative[1]	Source	Loading[a]	Activity[b]	Stabilization[c]
PGA C0	E.coli	80	100	1
PGA C1	E.coli	200	100	1400
PGA K1	K.citr.	80	100	7000
PGA K2	K. citr.	80	65	10000

[a]International Units of PGA (*see* Methods) per mL of derivative. Loading is the highest possible in each case using semipurified extract with approx the same degree of purity: 25%.

[b]Percentage of hydrolytic activity corresponding to soluble enzyme that has been immobilized.

[c]Ratio between the halflife time of each derivative and the one corresponding to soluble PGA from *E.coli* in experiments of irreversible thermal inactivation.

[1]Reference 5. PGA C0—one-point attached derivative; PGA C1—very intense multipoint covalent attachment prepared in the presence of a competitive inhibitor of PGA, penicillin G sulfoxide; PGA K1—as PGA C1; PGA K2—as PGA K1, but prepared in the absence of competitive inhibitor.

ated phenylacetic acid with 0.01*N* NaOH, 0.1*M* NaCl in a pH-stat (model TTT80 Radiometer, Denmark). Experiments were carried out at pH 8.0 at 25°C and using 20 mL of 30 m*M* of penicillin G in 0.1*M* NaCl, 50 m*M* phosphate. 1-4 IU of immobilized enzyme were used in each experiment. The presence of the very small amount of organic cosolvent (approx 50 μL) did not affect the observed reaction rates. On the other hand, high concentrations of phosphate buffer were used in this assay in order to avoid the generation of pH gradients inside the porous structure of these very active catalysts during this hydrolytic reaction. (An *International unit* of PGA is the amount of enzyme that hydrolyzes 1 μmol of penicillin G/min at pH 8.0 and 37°C.) A conversion factor of 1.7 was used to convert the actual activities measured at 25°C to IU (*14*).

Synthetic Reaction

A jacketed column was used as packed bed reactor. For kinetic analysis a small 3 mL bed of PGA-agarose derivative was used. The reaction medium was an equimolar mixture of thienyl acetic acid and 7-aminocephalosporanic acid (10 m*M*) dissolved in different water-DMF mixtures. After addition of the organic cosolvent, the pH was adjusted using acetate (25 m*M* in the total volume of mixture) and it is given as pH_{app}. Since the range of conversion analyzed was very low (lower than 10%), pH did not change during the synthetic reaction. In each case, different flowrates (0.1-2 mL/min) were used and reaction rates were extracted from representations of conversion percentage vs residence times. Substrates and

product (cephalothin) were identified and analyzed by HPLC using a KONIK Instruments (San Cugat, Spain) solvent delivery system with a SPECTRA PHYSICS SP 8450 detector, and a 250 * 4.6 mm RP-18 (5 μm) column (Spherisorb). Samples were eluted isocratically with 30% MeOH (v/v) in 0.067*M* $_kH_2PO4$, final pH_{app} 4.7, with a flowrate of 1.2 mL/min. The amount of product and reactants was determinated from calibration curves using stock solutions. For equilibrium experiments, large packed bed reactors (15 mL of PGA-agarose derivatives) were used. In this case a 100 mL solution of substrates was circulated through the reactor at 1.5 mL/min. After 50 mL had passed through the column, the solution was recirculated with continuous external adjustment of pH by using 1*N* HCl. When 100 μL of titrating solution had been added, the necessary amount of DMF (e.g., other 100 μL when concentration of cosolvent was 50%) was also added in order to keep constant the concentration of organic cosolvent. On the other hand, the use of concentrated titrant solutions avoided excessive dilution (<5%) of the reaction mixture.

Stability of PGA Derivatives in the Presence of DMF

Irreversible Inactivation

Samples of different PGA-agarose derivatives were suspended in different water-DMF mixtures and incubated at 4, 25, or 37°C. At different times, aliquots of these suspensions were withdrawn and assayed for hydrolysis of penicillin G in aqueous medium at pH 8.0 and 25°C, as described earlier. Water-DMF mixtures were prepared by mixing aqueous acetate buffer and cosolvent (final concentration of buffer was 25 m*M* in all cases) and pH_{app} of the mixtures was adjusted after addition of the cosolvent.

Total Inactivation

In other cases, the samples incubated in DMF were assayed in identical experimental conditions for the synthesis of cephalothin. In these cases, 3 mL columns were prepared and incubated in different water-DMF mixtures at different pH and temperatures. At different times, kinetic test of cephalothin synthesis (as the ones described in the previous paragraph) were performed in identical experimental conditions.

RESULTS AND DISCUSSION

Stability of Different PGA-Agarose Derivatives in 50% Water-DMF Mixtures

The time-courses of irreversible inactivation of different PGA-agarose derivates incubated in 50% water-DMF at pH 5.0 and at 25°C are given in Fig. 1.

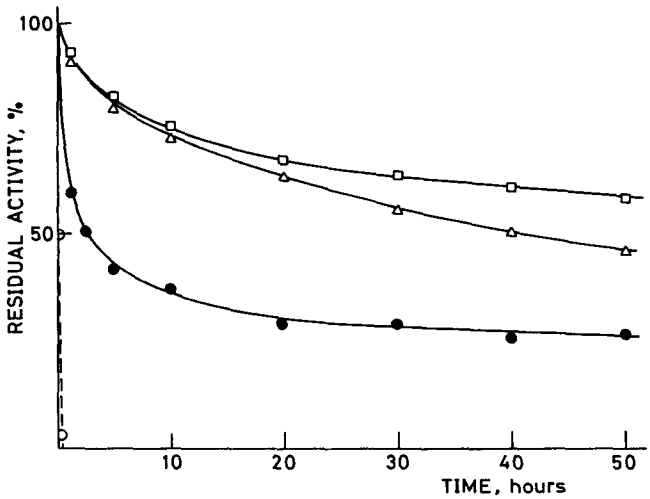


Fig. 1. Time-courses of irreversible inactivation of different PGA-agarose derivatives in 50% DMF. Derivatives were incubated in 50% DMF, pH 5.0 and 25°C and assay for penicillin G hydrolysis in fully aqueous medium at pH 8.0 and 25°C. *Open circles:* PGA C0. *Closed circles:* PGA C1. *Triangles:* PGA K1. *Squares:* PGA K2 (Properties and characteristics of each derivative are given in Table 1).

The presence of the organic solvent dramatically inactivates the enzyme structure. The pseudo half-life time of PGA C1 in 50% DMF-water at 25°C is the same (approx 3 h) as in fully aqueous medium at 60°C and pH 5.0.

Moreover, the order of stabilities is exactly the same as the one observed in experiments of irreversible thermal inactivation (Table 1). That is, the most thermally stable derivative PGA K2 was also the most stable one in the presence of organic cosolvents. Half-life times ranged from 1 min for PGA C0 derivative to 8 d for PGA K2 one. That means a stabilization of over 10,000-fold, which is similar to the stabilization observed in thermal inactivations (Table 1). This logical correlation between thermal and cosolvent inactivations has already been noted in a previous paper (7).

We also tested both total and irreversible inactivations of the most stable derivative PGA K2 in these DMF-water mixtures at 15 and 4°C. This derivative was fully stable (preserved more than 95% of hydrolytic [in aqueous medium] and of synthetic [in water-DMF] activity) after more than 1 mo of incubation under these experimental conditions (pH 5.0, 50% DMF and 4 or 15°C).

We also tested the effect of pH_{app} on the stability of PGA K2 derivative (Fig 2). We observed that stability presents a maximum at pHs around 7.0, at which the enzyme is 18-fold more stable than at pH 5.0.

From these results, PGA K2 derivative, a multipoint covalent attached derivative of PGA from *Kluyvera citrophila,* seems to be a good candidate to test the behavior of PGA as a synthetic catalyst in this type of DMF-water mixtures at a wide range of pH and temperatures (e.g., from 4 to 15°C and from pH 5.0 to 7.5). Under these conditions, catalyst inactivation is negligible

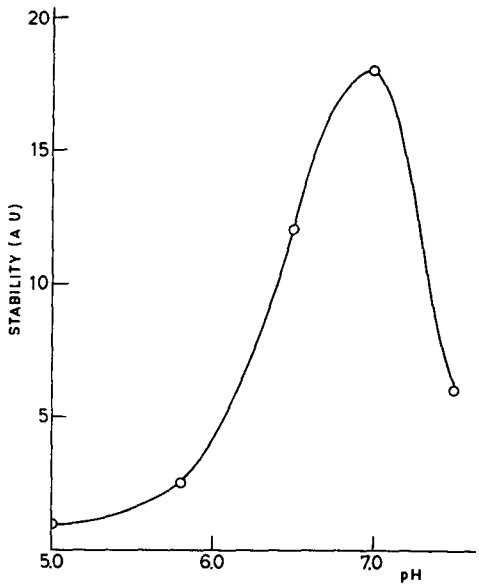


Fig. 2. Effect of pH on irreversible inactivation of PGA K2 derivative incubated in 50% DMF. Procedure is the same described in Fig. 2 but pH_{app} of the water-DMF mixture is different in each experiment. Stability Unit is the half-life time corresponding to pH 5.0.

and does not affect synthetic rates or apparent equilibrium yields. Obviously, the apparent yields and rates that we could obtain with a nonstabilized enzyme (e.g., PGA C0 at pH 5.0) could be subjected to very important artifacts because of catalyst inactivation (half-life time in 50% DMF, pH 5.0, 25°C is 1 min).

Since lower temperatures and higher pH increased the stability of PGA K2 derivatives, we also tested the stability of our PGA C1 (our most active derivative) at 4°C and pH 6.5 (approx the optimal conditions for both penicillin G and cephalothin synthesis as we shall comment on later in this paper). Under these conditions, the derivative was also extremely stable (more than 90% of catalytic activity was preserved after incubation in these conditions for 1 mo). Therefore, PGA C1 derivative also seems to present important perspectives for practical application because of its additional higher activity.

Cephalothin Yields in Equilibrium Controlled Synthesis

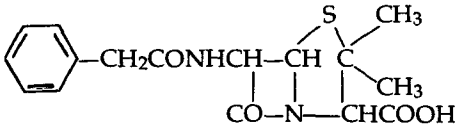
In Table 2 we represent some equilibrium synthetic yields corresponding to the synthesis of cephalothin, from thienyl acetic acid and 7ACA. under different experimental conditions as compared with the ones obtained for the synthesis of penicillin G (*9*). The values of synthetic yields were slightly lower for the synthesis of cephalothin, but both processes follow the same pattern. This seems to be logical if we have in mind that:

Table 2
Equilibrium Synthetic Yields of Cephalothin
and Penicillin G Catalyzed by PGA K2 Derivative
Under Different Experimental Conditions

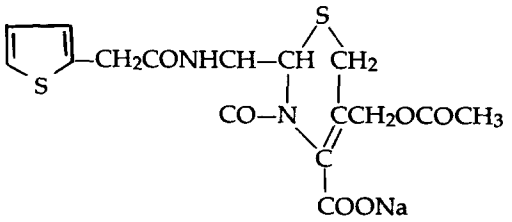
Solvent[1]	pH	[substrates][a]	Cephalothin[b]	Penicillin G[b]
water	6.0	10	2.5	3.0
50% glyc.	5.2	10	22	30
50 % DMF	6.0	10	55	65
50 % DMF	6.5	10	45	54
50 % DMF	6.5	20	60	70

Temperature: 4°C, 25 m*M* acetate.
[a]Equimolar concentrations of substrates (thienyl acetic acid and 7ACA for cephalothin synthesis and phenylacetic acid and 6 APA for penicillin G synthesis), m*M*.
[b]Equilibrium synthetic yield in percentage corresponding to substrate concentration.
[1]Glyc.: glycerol, DMF: N,*N*'dimethylformamide.

1. pKs of carboxyl and amine groups involved in these two reactions are very similar but slightly more unfavorable for cephalothin synthesis: pK, phenylacetic acid = 4.2, pK, TAA = 4.15, pK, 6APA = 4.6, and pK, 7ACA = 4.8 (*1*).
2. The intrinsic thermodynamic constant for the synthesis of an amide group between very similar structures must also be similar.



penicillin G



cephalothin

On the other hand, the dramatic effect of the presence of apolar organic cosolvents (e.g., DMF) on synthetic yields results evident: At pH 6.0 yield increases from 2.5 to 55 by the presence of 50% DMF. In this case, assumptions made in the introduction on the important beneficial role of the presence of the organic cosolvents appear to be clearly demonstrated.

The synthetic yields in Table 2 are the highest ones reported in literature. Interestingly, they were obtained under very mild conditions from the point of view of antibiotic stability (pH 6.5 instead of the most often used pH 5.0), and, using low equimolar concentrations of both reactants.

Evidently, this fact has been possible because we have prepared very stabilized derivatives of PGA and so we have been able to use more drastic conditions from the point of view of the organic cosolvent (50% DMF instead of 50% PEG or 20% acetone).

Moreover, higher yields would be obtained by using higher concentrations of substrates and/or an excess of one of the reactants, as we shall comment on later.

From the pKs of carboxyl and amine groups in different water-cosolvents mixtures (*9*) and from synthetic yields in Table 2, we can calculate the intrinsic thermodynamic constant, K_{TH}/a_w, as a function of the concentration of nonionic forms of reactants

$$K_{TH}/a_{water} = \frac{[\text{cephalothin}]}{[R - COOH] * [R' - NH_2]}$$

The mean value of K_{TH}/a_w is $950M^{-1}$ at 4°C. This value seems to have a real thermodynamic significance because:

1. Values calculated by using the different set of data were similar (±5% of error), hence they are independent from initial substrate concentrations and from the solvents used. It seems that problems of inhibition by product (which may be responsible for artifacts in synthetic yields) are not present in these experimental conditions.
2. Our PGA K2 derivative was extremely stable in all these experimental conditions. So, inactivation of the catalyst is negligible during all the time-courses of the synthesis. Inactivation of the catalyst also may be a very important source of artifacts in synthetic yields even working at laboratory scale. Kasche et al. (*2*) found differences in apparent equilibrium yield of penicillin G by using different derivatives. Similarly, Morihara and Oka reported an increase of apparent synthetic yields in reactions catalyzed by chymotrypsin with enzyme concentration (*12*). These two facts, meaningless from a thermodynamical point of view, may be mainly caused by inactivation of the catalyst during the course of the reaction.

Synthetic Activity of PGA K2 Derivative

In Fig. 3, we compare the reaction rates of cephalothin synthesis in 50% DMF with the ones corresponding to penicillin G synthesis. Cephalothin synthesis at pH 5.0 could not be studied because of the lack of solubility of 7ACA under these experimental conditions. Both curves follow a cleary parallel course, being in all cases the rates of cephalothin synthesis 30–35% of the rates of penicillin G synthesis. This decrease of activity for cephalothin synthesis agrees with previous results reported in literature for specificity of PGA from *E. coli* measured in the case of peni-

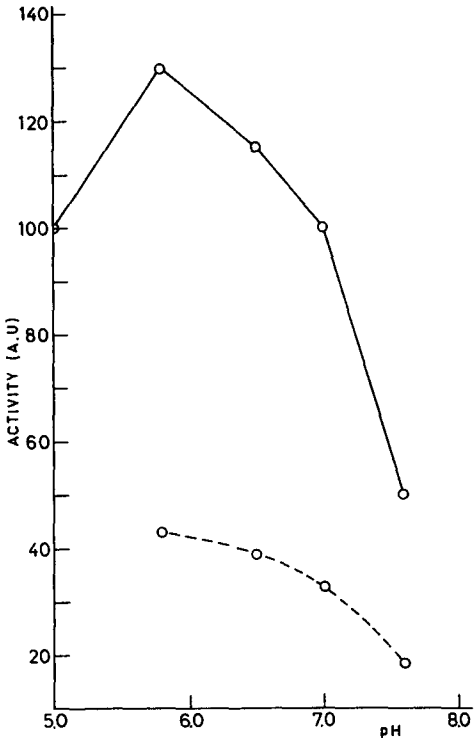


Fig. 3. Initial reaction rates for cephalothin and penicillin G synthesis catalyzed by PGA K2 derivative in 50% DMF at different pH values. Experimental conditions: 10 m*M* of both substrates (acyl donor and nucleophile), 4°C, 25 m*M* acetate buffer in 50% water-DMF mixtures. (For details, *see* Materials and Methods.) 100% of synthetic activity is the one obtained for synthesis of penicillin G at pH 5.0 in fully aqueous medium and this is exactly the same obtained with 50% DMF. *Straight lines:* penicillin G synthesis. *Broken lines:* cephalothin synthesis.

cillin G or cephalothin hydrolysis (*13*). In this same way, we tested that specificities of these two PGAs are similar (*14*).

Since 100% of synthetic activity corresponds to the synthesis of penicillin G in a fully aqueous medium at pH 5.0, the presence of the organic cosolvent does not exert very dramatic changes in both synthetic rates (penicillin G and cephalothin). For example, activity at pH 5.0 and 50% DMF was exactly the same and activity was superior to 100% at pH 5.8.

In a previous paper (*8*), we demonstrated, by using a hydrolytic reaction, that organic cosolvents act as competitive inhibitors of PGA. The hydrophobic character of the acyl donor chain must imply a very important role of hydrophobic interactions in enzyme-substrate recognition. Hence, the organic cosolvent could also adsorb on the hydrophobic pocket of the enzyme and compete with the substrate. This deleterious effect on the reaction rates should be even more clear in the case of syn-

thetic reaction and, however, we have now seen a very slight effect of the organic cosolvent and even an apparent beneficial effect in some cases. We must assume that the presence of organic cosolvent also increases the concentration of nonionic form of acyl donor and evidently this is the only form able to react with PGA to yield the enzyme-substrate covalent intermediate. For this reason, parallel with its effect on thermodynamic yield, the presence of organic cosolvent also has an important profitable effect on the reaction rates. Therefore, the shape of the curves represented in Fig. 3 may reflect these two opposite effects: increase of the active form of the acyl donor substrate with respect to the one in water at pH 5.0 (this effect is very important at low pHs) and inhibitory effect of the cosolvent (which becomes more important at high pHs where the concentration of nonionic forms of acyl donor decreases and becomes similar to the one in water at pH 5.0). Evidently, a more detailed kinetic study of this synthetic reaction is necessary and is now being performed in our laboratory.

Quantitatively, synthetic reaction rates are quite slow. By using 10 m*M* of substrates, at pH 6.0, 50% DMF and 4°C, we have calculated a rate of production of antibiotic of 5g/h/L of catalyst. In our concluding remarks, we consider the possibilities for increasing the volume activity of these PGA-agarose derivatives.

From results presented up to now (stability of the catalyst, synthetic yields, reaction rates) and from stability of the antibiotic cephalothin (it increases as pH approaches neutrality), it seems that optimal conditions previously found for penicillin G synthesis (*9*) (50% DMF, pH 6.5, and 4°C) apply very well to the synthesis of cephalothin. For example, synthetic yields and rates are maximal at pH 6.0, but enzyme and antibiotic stability are maximal at pH 7.0. So, pH 6.5 appears to be a good compromising solution.

However, 7ACA presents important problems of solubility and therefore slight variations in these optimal conditions must be introduced if we wish to synthesize high antibiotic concentrations.

Solubility of 7ACA in Water and in Water-DMF Mixtures: Synthetic Reactions with High Substrate Concentrations

We have been unable to dissolve more than 5 m*M* of 7ACA in water-DMF at pH 5.0. Up to 10–20 m*M* were dissolved in these water-organic solvent mixtures at higher pHs, but more than 50 m*M* were only dissolved at pHs greater than 7.2. High solubility of 7ACA is only reached at a too high pH value, which is unfavorable from a thermodynamic and kinetic point of view.

In order to test this synthetic reaction by using high concentrations of reactant (which could be very important from an industrial perspective) we performed this reaction by using "dynamic conditions." That is, we started the reaction with 50 m*M* 7ACA and an excess (200 m*M*) of thienyl-

acetic acid at high pH (7.2) with the solution recirculating through the column of PGA K2 derivative at 4°C and external control of the pH. In the first stages of the reaction, pH was maintained at 7.2, but when synthesis was developing, pH was allowed to fall down to "optimal conditions." When conversion (referred to 7ACA) was 25%, the pH was allowed to fall down to 7.0; when conversion reached 50%, pH was allowed to fall down to 6.7; and after conversion reached 75%, pH was allowed to fall down to 6.4 (already within the range of optimal conditions). In this way, we were able to reach a final synthetic yield of more than 95% without problems of solubility of substrate. This corresponds to a final antibiotic concentration of 2% w/v, which falls within a very interesting industrial range. This yield is clearly superior to the one obtained in case of use "static conditions" (e.g., using pH 7.2 during all the course of the reaction synthetic yield resulted approx 80%).

CONCLUDING REMARKS

We would like to make some interesting observations.

1. Using the most simple synthetic strategy (equilibrium controlled synthesis in monophasic systems) we were able to obtain very high synthetic yields of an interesting antibiotic, cephalothin.
2. These high synthetic yields were obtained under very mild experimental conditions—50% DMF in almost neutral media, in which the antibiotic, the antibiotic nucleus 7ACA, and the catalyst (PGA K2 derivative) were very stable.
3. As expected, the results obtained for the synthesis of cephalothin were quite similar to the ones previously found for the model synthesis of penicillin G. Optimal conditions for these two reactions had been taken from a compromising and integrated solution based on an overall consideration of synthetic yields, enzyme activity, enzyme stability, antibiotic stability, and so on.
4. Now in the synthesis of cephalothin, because of problems of substrate solubility, those optimal conditions were slightly varied when we intended to handle high substrate concentrations. In this situation, a "dynamic strategy" was chosen in order to prevent substrate precipitation during the time-course of the reaction and in order to achieve very important final synthetic yields (very close to 100%).
5. The use of previously stabilized penicillin G acylase derivatives becomes necessary to test and to scale up this type of synthesis because of the dramatic effects organic cosolvents exert on enzyme stability. Under the optimal synthetic conditions, our

best derivatives (multipoint covalent attached derivatives between PGA from *K. citrophila* and agarose (aldehyde) gels), resulted in being fully stable. They preserved more than 95% of activity after incubation in 50% of DMF, at 4°C, in the range of pHs 6.4–7.2 during more than 2 mo.

6. We have calculated a productivity of 4.3 Tm of cephalothin/yr by using a catalytic reactor containing 100 L of our PGA K2 derivative. However, in our opinion there are a number of ways to improve the volume activity of PGA derivatives in this synthetic reaction at least by one order of magnitude and so we could already fall within a very interesting range of industrially useful conditions. Some of those ways are: (a) use of agarose gels that are of smaller pore size (e.g., 14 and 16% agarose gels), which are now being produced by HISPANAGAR S.A. (Burgos, Spain), (b) use of more purified extracts of the enzyme from *K. citrophila*, and (c) use of dynamic conditions during the complete synthetic reaction (in the same way that we performed high concentrations).

ACKNOWLEDGMENTS

We would like to thank Boehringer Mannheim GmbH (Penzberg, FRG), Antibioticos S.A. (León, Spain), and Cipan S.A.R.L. (Lisbon, Portugal) for their generous gifts of Penicillin G acylase from *E. coli*, penicillin G acylase from *K. citrophila*, and 7-aminocephalosporanic acid. We are also very grateful to Victor M. Fernandez (Instituto de Catalisis, Spain) and Alan Russell (University of Pittsburgh, PA) for very helpful discussions, and to Maria del Carmen Ceinos for excellent technical assistance. This work was supported by the Spanish CICYT (Project no.: BIO88-276-01) and by the Commission of the European Communities (Biotechnology Action Programme, Contract no.: 395.E (JR).

Part of this work is included in the Spanish Patent Application No. 8904193, 1989.

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