CONVERSION OF PENICILLIN N TO CEPHALOSPORIN(S) BY CELL-FREE EXTRACTS OF *CEPHALOSPORIUM ACREMONIUM*

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SUMMARY

A procedure has been developed for preparing cell-free extracts of *Cephalosporium acremonium* that are capable of synthesizing cephalosporin(s). Protoplast pellets, obtained by treating 60-hr-old mycelia with *Cytophaga* lytic enzyme L_1 plus Zymolyase from *Arthrobacter*, were gently homogenized in a Teflon homogenizer, and the product was centrifuged to provide cell-free extracts. The reaction mixture contained an energy-generating system, mannitol, $MgSO_4$, KCl and Tris buffer (pH 7.2). The mixture was shaken at 25°C for 5 hr and then assayed directly with a mutant of *Escherichia coli* supersensitive to β-lactam antibiotics. The principle antibacterial product(s) was resistant to penicillinase but was destroyed by "cephalosporinase." An energy-generating system, although not required, stimulated production; intensive shaking (250 rev/min), however, was necessary. The production of cephalosporin was inhibited by KCN, but unaffected by Millipore filtration, cycloheximide or a mixture of the constituent amino acids. Production was markedly stimulated by penicillin N but not by penicillin G or 6-aminopenicillanic acid. The addition of penicillinase to the cell-free extract eliminated cephalosporin production. The results indicate that the cell-free extract can convert endogenous and exogenous penicillin N to cephalosporin(s). Such ring expansion is known in synthetic chemistry but has never before been observed in a biological system.

INTRODUCTION

The ability of protoplast lysates from *Cephalosporium acremonium* to incorporate labeled valine into penicillin N and cephalosporin C was mentioned in a review by Abraham (1) in 1974. Such a cell-free system would be useful in the study of β-lactam antibiotic biosynthesis because it contains no barriers

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to permeability. Using the cell-free system from *C. acremonium*, Fawcett *et al.* (2) found that tritium-labeled δ-(L-α-amino-adipyl)-L-cysteinyl-D-valine was incorporated into penicillin N. In our laboratories, Bost and Demain (unpublished results) have been able to convert [^{14}C]-L-valine to penicillin N by using a similar lysate, but cephalosporins were not formed. Our continued efforts to improve the cell-free system have yielded an enzyme preparation capable of synthesizing cephalosporin(s) in amounts large enough to be detected by direct bioassay with a mutant of *Escherichia coli* that is supersensitive to β-lactam antibiotics.

This report describes the activity of our system. Of great interest is its apparent ability to convert penicillin N to cephalosporin(s). Although chemical conversion of penicillins to cephalosporins is known (3), this is the first report of ring expansion in a biological system.

MATERIALS AND METHODS

Media and conditions for growing *C. acremonium* CW 19 have been described (4). Cultures were incubated at 25°C in 250-ml Erlenmeyer flasks containing 40 ml of chemically defined medium and shaken on a rotary shaker at 250 rpm (2-in diameter orbit).

The mycelium harvested at 60 hr was filtered and washed three times with 20 ml of distilled water. The damp-dry mycelium from one flask was resuspended in 10 ml of McIlvaine's citrate-phosphate buffer (pH 7.3) (5) containing 0.01 M dithiothreitol and then incubated for 1 hr at 28°C with shaking at 120 rpm. The mycelium was filtered and washed and then resuspended in 10 ml of McIlvaine's buffer (pH 7.3) containing 1.0 M NaCl, 0.02 M $MgSO_4$ and 40 mg of lytic enzyme L_1 from *Cytophaga* plus 40 mg of Zymolyase from *Arthrobacter* (6). The suspension was incubated at 28°C for 3 hr and shaken at 120 rpm. The resulting protoplast suspension was centrifuged at 800 × *g* for 10 min, and the pellet was washed twice with 20 ml of Tris buffer (pH 7.2, 0.05 M) containing 0.65 M mannitol, 0.01 M $MgSO_4$ and 0.01 M KCl. The protoplast pellet was gently homogenized in a Teflon homogenizer at 4°C. After adding 2 ml of Tris buffer, we centrifuged the suspension at 1000 × *g* for 10 min. About 2.5 ml of cell-free extract were obtained per shake flask.

To 1 ml of cell-free extract in a 10-ml Erlenmeyer flask we

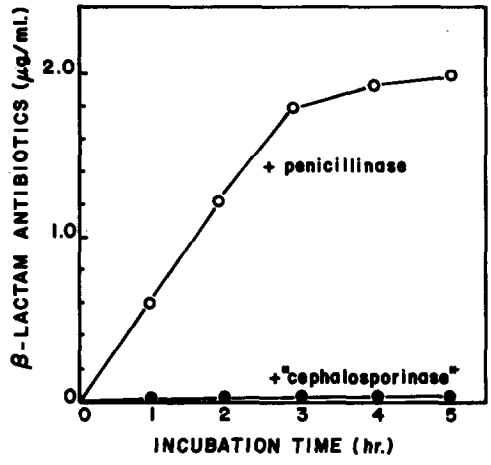

Fig. 1. The synthesis of cephalosporin(s) by the cell-free system from *C. arcremonium*.

The reaction mixture, in a final volume of 1 ml in a 10-ml Erlenmeyer flask, contained an energy-generating system, (5 μmoles of ATP, 10 μmoles of PEP and 100 μg of pyruvate kinase), 0.01 M $MgSO_4$, 0.01 M KCl, 0.65 M mannitol and Tris buffer (pH 7.2, 0.05 M). The flask was shaken at 25°C on a rotary shaker at 250 rpm. Bioassay was conducted with *E. coli* ESS in the presence of penicillinase or "cephalosporinase."

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added 5 μmoles of adenosine triphosphate (ATP), 10 μmoles of phosphoenolpyruvate (PEP) and 100 μg of pyruvate kinase. The final pH of the cell-free system was adjusted to 7.2. The mixture was incubated for 5 hr at 25°C on a shaker at 250 rpm.

A mutant of *E. coli* (designated ESS) was isolated, which was supersensitive only to β-lactam antibiotics (7). This mutant was about equally sensitive to cephalosporin C, deacetylcephalosporin C, deacetoxycephalosporin C and penicillin N. Bioactivity was assayed by placing paper discs on plates of Antibiotic Medium No. 5 (Difco Laboratories, Detroit, MI) seeded with *E. coli* ESS. After incubation at 37°C for 15 hr, zones of inhibition were measured. The amount of β-lactam antibiotic produced in the cell-free system was estimated by the increase in the size of the zone during the 5-hr incubation; cephalosporin C was used as the standard.

Cephalosporin C was a gift from Dr. C.A. Claridge of Bristol Laboratories (Syracuse, NY). Deacetylcephalosporin C, deacetoxycephalosporin C and penicillin N were obtained from Dr. R.L. Hamill of the Lilly Research Laboratories (Indianapolis, IN). The penicillin N sample was only 20% pure. All figures in this paper refer to the actual content of penicillin N, not to total solids in the preparation. "Cephalosporinase," prepared from sonically ruptured cells of *Enterobacter cloacae*, was obtained from Dr. L. Fare of Smith, Kline and French Laboratories (Philadelphia, PA). This suspension attacks not only the three cephalosporins, but also penicillin N. Dithiothreitol, ATP, PEP and pyruvate kinase were purchased from Sigma Chemical Co. (St. Louis, MO). Lytic enzyme L_1 from *Cytophaga* was obtained from BDH Biochemicals (Poole, England). Zymolyase was obtained from Kirin Brewery Co., Ltd. (Tokyo, Japan). Penicillinase was purchased from Difco Laboratories.

Table 1. Effect of Millipore filtration of the lysate on the synthesis of cephalosporin(s).

Condition	Cephalosporin (μg/ml)
Protoplast lysate	1.5
Protoplast lysate after Millipore filtration	1.4

Reaction conditions were the same as those described in Fig. 1.

Table 2. Effect of various factors on the synthesis of cephalosporin(s) in the cell-free system.

Condition	Cephalosporin (μg/ml)
Complete system	1.6
Minus energy-generating system	0.8
Plus 1 mM KCN	0
Shaking decreased to 120 rpm	0
Plus 100 μg of cycloheximide/ml	1.5

Reaction conditions were the same as those described in Fig. 1.

RESULTS

As shown in Fig. 1, the protoplast lysate synthesized a β-lactam antibiotic(s) that was resistant to penicillinase but sensitive to "cephalosporinase." Production of the cephalosporin(s) was linear for 3 hr and reached its maximum level at 5 hr.

The lysate contained cell debris and oily particles that were not removed by centrifugation at 1000 × *g* for 10 min. To

remove these materials, the lysate was filtered through a size RA Millipore filter. The activity of the cell-free system was not decreased by filtration (Table 1), showing that the intact cells were not responsible for the activity.

An energy-generating system was required for maximum cephalosporin production (Table 2). The addition of KCN (1 mM) completely inhibited cephalosporin production, in spite of the presence of ATP and an energy-generating system. Furthermore, reducing the agitation speed from 250 to 120 rpm also resulted in complete inhibition. Table 2 also shows the lack of effect of cycloheximide, indicating that protein synthesis is not required during the 5-hr incubation.

C. acremonium normally produces cephalosporins and penicillin N in fermentations from L-α-aminoadipic acid, L-valine and L-cysteine. As shown in Table 3, these amino acids had no effect on cephalosporin production in our system. This finding suggested that either our preparation contained excess amino acids or it was converting a late intermediate to cephalosporin(s). It seemed possible that such a late intermediate could be the penicillin N present in the cell-free extract. To investigate this possibility, we added a crude preparation of penicillin N to our system. As shown in Table 4, it markedly stimulated cephalosporin production. Conversely, penicillin G and 6-aminopenicillanic acid showed no stimulatory activity. Because the penicillin N sample used in our experiments was only 20% pure, we treated the preparation with penicillinase until its antibacterial activity against *E. coli* ESS was destroyed. This treated sample totally inhibited cephalosporin production (Table 5). This inhibition was evidently due to carryover of penicillinase, which destroyed endogenous penicillin N. When the cell-

Table 3. Lack of stimulation of cell-free cephalosporin production by amino acids.

Condition	Cephalosporin (μg/ml)
Complete system	1.5
Complete system plus amino acid mixture	1.6

Each constituent amino acid (L-α-aminoadipic acid, L-cysteine and L-valine) was added to the complete system at a concentration of 5 μmoles/ml. Reaction conditions were the same as those in Fig. 1.

Table 4. Penicillin N stimulation of cephalosporin production in the cell-free system.

Condition	Cephalosporin (μg/ml)
EXPERIMENT I:	
Complete system	1.2
Plus penicillin N (10 μg/ml)	3.2
Plus penicillin N (10 μg/ml) and 1 mM KCN	0
Plus penicillin N (10 μg/ml) and shaken at 120 rpm	0
EXPERIMENT II:	
Complete system	2.0
Plus penicillin G (10 μg/ml)	1.7
Plus 6-amino penicillanic acid (10 μg/ml)	1.8

Reaction conditions were the same as those described in Fig. 1.

Table 5. Inhibition of cephalosporin production by penicillinase treatment of penicillin N or the cell-free extract.

Condition	Cephalosporin (μg/ml)
Complete system	1.3
Plus penicillin N (10 μg/ml)	2.3
Plus penicillinase-treated penicillin N (10 μg/ml)*	0
With cell-free extracts pretreated with penicillinase†	0

Reaction conditions were the same as those described in Fig. 1.

* Ten μliters of penicillinase were added to 1 ml of penicillin N (100 μg/ml), and the solution was incubated at 37°C for 1 hr. A 0.1-ml aliquot of the sample was added to the cell-free system.

† Five μliters of penicillinase were added to 1 ml of the cell-free extract, and the solution was incubated at 37°C for 1 hr.

free extracts were pretreated with penicillinase to inactivate endogenous penicillin N, they could not synthesize cephalosporin(s).

DISCUSSION

Production of antibiotic(s) by the cell-free system was assayed directly with *E. coli* ESS, which is supersensitive to β-lactam antibiotics. Since penicillinase specifically inactivated penicillin N, cephalosporins could be detected by a bioassay in which strain ESS was used in a plate containing penicillinase. As shown in Fig. 1, cephalosporin(s) was produced in our cell-free system. The lack of stimulation by the precursors (α-aminoadipate, cysteine and valine) could have been caused by the presence of saturating concentrations of these amino acids in the

cell-free extract. Alternatively, the extract may only catalyze one or a few terminal reactions. At any rate, the marked stimulation by penicillin N and the elimination of activity in the cell-free extract by penicillinase indicate that the conversion of penicillin N to cephalosporin(s) is the activity being measured in our system. Although chemists have been able to expand the penicillin ring system into that of the cephalosporins, ring expansion under biological conditions has never been observed before. Indeed, many investigators have considered penicillin N and cephalosporins to be end-products of a branched pathway (8).

Because neither penicillin G nor 6-aminopenicillanic acid had any stimulatory effect, it appears that we are dealing with specific enzyme reactions that require penicillin N as a substrate. The complete inhibition of the process by KCN or decreased agitation (Tables 2 and 4) suggests the involvement of an oxygenase in the conversion of penicillin N to cephalosporin. The chemical transformation of penicillin to cephalosporin achieved by Morin and coworkers (3) involved activation of the sulfur atom by oxidation, cleavage of the C_2-sulfur bond by introducing a double bond and reclosure of the ring to cephalosporin. By analogy with the chemical reaction, the first step of the bioconversion probably involves an oxygenase that requires a high degree of oxygen transfer. The fact that an increase in oxygen tension can increase production of cephalosporin C and reduce production of penicillin N by intact cells (9, 10) is consistent with this hypothesis.

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