FURTHER STUDIES ON MICROBIOLOGICAL RING-EXPANSION OF PENICILLIN N

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The rate of microbiological ring-expansion of penicillin N to deacetoxycephalosporin C using protoplast lysates of the antibiotic-negative mutant *Cephalosporium acremonium* M-0198 has been increased some 70-fold over that of our earlier system. We confirmed the stimulatory effects of $FeSO_4$ and ascorbate described by HOOK *et al.* (Biochem. Biophys. Res. Commun. 87: 258, 1979); the optimum concentrations found were 0.04 mM $FeSO_4$ and 0.67 mM ascorbate. Adenosine triphosphate concentration was lowered to 0.83 mM; phosphoenolpyruvate and pyruvate kinase were eliminated. The optimum pH and temperature for the reaction were 7.2 and 25°C, respectively. α-Ketoglutarate and $MnCl_2$ showed no marked effect on the reaction, $MgSO_4$ and KCl were mildly stimulatory, and $CuSO_4$ and $ZnSO_4$ were very inhibitory. Penicillin N was optimal at a concentration of 0.07 mM. Specific ring-expansion activity reached its peak 13 hours after growth ceased and then disappeared rapidly.

In our earlier studies[1,2], we demonstrated the enzymatic conversion of penicillin N to deacetoxycephalosporin C using cell-free extracts of *Cephalosporium acremonium* CW-19 and its antibiotic-negative mutant M-0198. The extracts catalyzing this ring-expansion reaction were prepared by lysing *C. acremonium* protoplasts[3,4]. The activity was relatively weak, however, and other investigators have had difficulty carrying out the reaction. HOOK *et al.*[5] discovered that a cofactor mixture of ferrous sulfate, ascorbate, and α-ketoglutarate (α-KG) stimulated the reaction. We have further optimized the cofactor requirements and markedly increased the conversion's rate and extent. These experiments are described in the present paper along with additional data concerning the ring-expansion reaction. We expect that, with these modifications, the system will be widely used by antibiotic investigators.

Materials and Methods

Culture

C. acremonium strain M-0198 (NRRL 11418), the β-lactam-negative mutant of strain CW-19[2], was used. Maintenance and growth methods were as previously described[6] with the exception noted in the next paragraph.

Growth

In previous studies[2], individual fermentation flasks were prepared and inoculated with 5 ml of seed culture. In the present work, the procedure was modified to improve reproducibility between flasks. The basal medium and sugars were autoclaved (separately) and combined in a large sterile flask, and the inoculum added. After thorough mixing, individual 45-ml portions were aseptically

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distributed into sterile 250-ml flasks. The inoculum was grown in three separate flasks for 4 days and combined before use. The fermentation was routinely followed by absorbance in the Klett-Summerson colorimeter, using the red filter, after diluting the whole broth with 0.01 N HCl (to dissolve $CaCO_3$ carried over from the seed medium) to a figure below 100 Klett units. The fermentation broth's pH value was determined after filtration through filter paper (Schleicher and Schuell, No. 595). In certain fermentations, we determined mycelial wet weight after growth. The flask contents were filtered through paper (S & S, No. 595) in a Buchner funnel. The mycelia were washed twice, each time with 20 ml of distilled water, and weighed. We found a direct relationship between absorbance and mycelial wet weight: 1 g wet mycelium/liter equals 42 Klett units.

Preparation of cell-free extracts

The method of YOSHIDA *et al.*[2] was modified slightly, as follows. After being treated with lytic enzymes, centrifuged, and washed, the pellet was suspended in 0.05 M Tris-HCl buffer (pH 8.0) containing 0.01 M KCl and 0.01 M $MgSO_4$ (Tris-HCl buffered salts). This produced a final pH of 7.2 for the cell-free reaction mixture. After the lysate was incubated in the freezer (-20°C, 60 minutes), the suspension was homogenized with a steel spatula, and centrifuged at $3,000 \times g$ for 10 minutes. The supernatant fluid (*i.e.*, cell-free extract) was used immediately or stored at -65°C until used.

Ring-expansion reaction

The initial procedure was slightly modified from YOSHIDA *et al.*[2] Energy-supplying system and penicillin N (0.08 ml each) were added to 0.84 ml cell-free extract in a 10-ml Erlenmeyer flask to give a total volume of 1 ml. The final concentrations of the energy-supplying system's components were 4.2 mM adenosine triphosphate (ATP; Sigma), 8.3 mM phosphoenolpyruvate (PEP; Sigma), and 80 μg/ml pyruvate kinase (PK; Sigma). The penicillin N preparation was impure and labile; its active concentration was determined by bioassay. The final concentration of penicillin N in the assay was approximately 25 μg/ml or 0.07 mM. The cell-free extracts usually contained $4 \sim 10$ mg protein/ml. As described below, the extract could be diluted up to 16-fold before use, with the improved system. In these cases, the diluent was 0.05 M Tris-HCl buffered salts (pH 7.2). The reaction mixture was incubated 1 hour on a shaker at 250 rpm and 25°C. At various times (including zero time) 25-μl samples were removed, placed on paper discs (S & S, No. 740-E, 6.35 mm diameter), and assayed as described below. Two control discs were always included. On one disc, we placed the entire cell-free reaction mixture before penicillin N addition. This disc produced no inhibitory zone at all; if a zone were seen, it would mean that the antibiotic-negative *C. acremonium* mutant had reverted and produced antibiotic during growth. On the other control disc, we placed penicillin N at the same concentration as in the cell-free reaction. An inhibitory zone should appear on the plate without penicillinase, but not on the penicillinase-containing plate. This control ensures that enough penicillinase is present to destroy any residual penicillin N that diffuses into the agar. Thus, zones on the penicillinase-containing plate should reveal only deacetoxycephalosporin C activity.

Bioassay

Pseudomonas aeruginosa Pss, the assay organism, is a β-lactam-supersensitive mutant that is equally sensitive to penicillin N, cephalosporin C, and deacetoxycephalosporin C. Because *P. aeruginosa* frequently reverts to normal sensitivity, special precautions were taken. The reverted population was recloned on agar plates. After a particular colony was determined still to be supersensitive, it was spread on two agar plates (Difco Antibiotic Medium No. 5), grown, and the cells harvested and washed twice with saline, then suspended in 20 ml of 50% (v/v) glycerol. The suspension was divided into a large number of sterile vials at 0.5 ml per vial and frozen at -65°C until used. For each assay, a tube was thawed and its contents suspended in molten assay agar at a concentration of 0.1% (v/v). To assay total β-lactams, no penicillinase was present in the agar. To assay deacetoxycephalosporin C, penicillinase (Difco) was added at 500 units/ml agar (half-strength seeded Difco Antibiotic Medium No. 5). The plates contained 8 ml agar, and cephalosporin C (0.3 to 30 μg/ml) was used as a standard. Discs containing a 25-μl sample or standard were placed in an empty Petri dish which was kept in the freezer until all the samples had been taken. All discs were then exposed to chloroform vapors for 1 hour in a desiccator kept at 4°C, to destroy any contaminating organisms possibly introduced dur-

ing the lytic enzyme treatment. The discs were then placed on the assay plates. Each sample was applied to four discs so that two discs could be placed on each type of assay plate. Plates were incubated overnight at 37°C. Any activity present at zero time was subtracted from all subsequent samples.

Protein determination

Protein in cell-free extracts was measured by the method of BRADFORD[7]. Bovine serum albumin (Sigma) was used as standard.

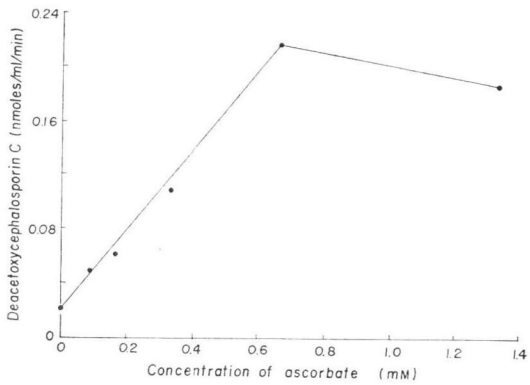
Results

Optimization of Cofactor Concentrations

HOOK *et al.*[5] reported that a mixture of 3.8 mM ascorbate, 0.075 mM $FeSO_4$, and 3.8 mM α-KG stimulated ring-expansion when added to our previous reaction mixture of penicillin N, ATP, PEP, and PK in Tris-HCl buffered salts (final pH 7.2). We confirmed these findings and in preliminary experiments observed even better reaction rates when the mixture of α-KG, ATP, PEP, and PK was eliminated. (Later, a low ATP level was found to be stimulatory.) Using a concentration of 0.08 mM $FeSO_4$, we determined the optimum ascorbate concentration to be 0.67 mM (Fig. 1). The optimum $FeSO_4$ concentration was determined next, holding the ascorbate constant at 0.67 mM; $FeSO_4$ was optimal at 0.04 mM (Fig. 2). A marked synergy was observed with the optimum concentrations of the $FeSO_4$ and ascorbate supplements. Reactions conducted without cell-free extract or with boiled cell-free extract showed no product formation. Ascorbate and $FeSO_4$ increased the activity so much that cell-free lysates could be diluted up to 16-fold and still show good activity. Production rate was proportional to protein concentration up to 1 mg protein/ml reaction mixture.

Fig. 1. Effect of ascorbate concentration on ring-expansion activity.

Ferrous sulfate was present at 0.08 mM. ATP, PEP, and PK were omitted. The protein concentration in the cell-free extract was 0.95 mg/ml.



Effects of ATP, PEP, and PK

Using the optimum concentrations of $FeSO_4$ and ascorbate, we determined the effects of ATP and PEP plus PK on conversion activity. ATP was stimulatory at 0.83 mM and became inhibitory at higher concentrations (Fig. 3a). On the other hand, a mixture of 0.83 mM PEP plus 8 μg/ml PK showed only a slight stimulation before the combi-

Fig. 2. Effect of ferrous sulfate concentration on ring-expansion activity.

Ascorbate was present at 0.67 mM. ATP, PEP, and PK were omitted. The protein concentration in the cell-free extract was 0.48 mg/ml.

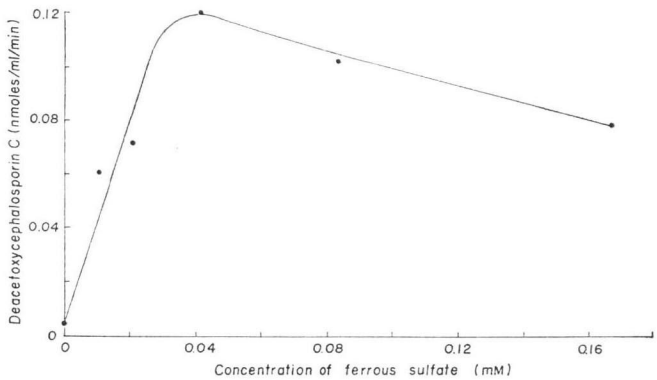
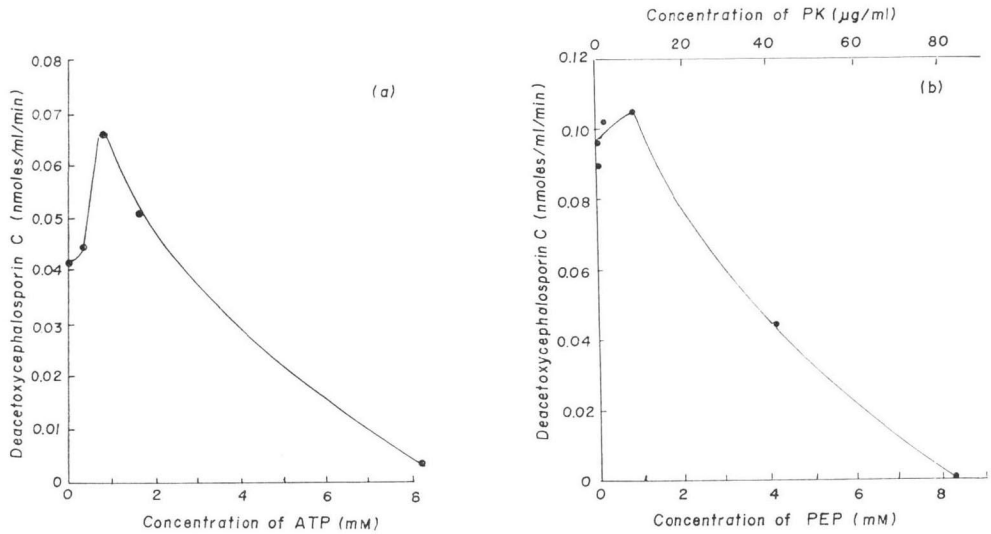


Fig. 3. Effect of concentration of (a) ATP and (b) its regenerating system (PEP and PK) on ring-expansion activity.

Ascorbate was present at 0.67 mM and $FeSO_4$ at 0.04 mM. In (a), no PEP or PK was present. In (b), ATP was present at 0.83 mM. The protein concentration in the cell-free extract was 0.48 mg/ml.



nation became inhibitory (Fig. 3b). We decided to omit the PEP and PK for the rest of the study and to include ATP at 0.83 mM, which is 20% of the level used by YOSHIDA *et al.*[2]

$MgSO_4$ and KCl had been used in the cell-free reaction at a concentration of 10 mM each. These salts were examined together from 1 mM to 400 mM in the ascorbate-$FeSO_4$-ATP system. The 10-mM concentration of each supported the highest activity, which was about 25% greater than at the lowest salts concentration. Concentrations of 20 mM and higher inhibited the reaction.

Effects of Other Factors

The optimum pH of the ring-expansion reaction is 7.2 (Fig. 4). The optimum temperature is 25°C (Fig. 5). Both pH 7.2 and 25°C were used in our previous studies[2]. α-KG was examined at concentrations from 0 to 1.3 mM. Little or no effect was observed at concentrations up to 0.32 mM; higher concentrations were mildly inhibitory. $CuSO_4$, $ZnSO_4$, and $MnCl_2$ were each tested at 0.08 mM in the ascorbate-$FeSO_4$-ATP system for their effects. Ring-expansion activity was completely inhibited by $CuSO_4$ and $ZnSO_4$, but unaffected by $MnCl_2$. The ring-expansion reaction's maximum rate was obtained at a concentration of 0.07 mM penicillin N, which is equivalent to 25 μg active material/ml (Fig. 6). This substrate concentration is convenient to use, because 500 units penicillinase/ml agar medium can destroy this concentration without destroying any of the deacetoxycephalosporin C produced. Increasing the substrate concentration would cause penicillin N zones to appear on penicillinase-containing plates, which would complicate the deacetoxycephalosporin C assay.

We previously reported[2] that cell-free extracts were stable when frozen at −20°C. In the present study, we found the ring-expansion activity to be completely stable when lyophilized.

Identity of the Product

In our earlier work[2], our cell-free reaction in the presence of ATP and PEP plus PK produced deacetoxycephalosporin C. To determine whether the same product is made in the ascorbate-$FeSO_4$-

Fig. 4. Effect of pH on the ring-expansion activity.

Ascorbate, $FeSO_4$, and ATP were present at 0.67 mM, 0.04 mM, and 0.83 mM, respectively. PEP and PK were not present. Between pH 5.2 and 7.2, the buffer was 0.05 M phosphate containing $MgSO_4$ and KCl. Between pH 7.2 and 9.2, the buffer was 0.05 M Tris containing $MgSO_4$ and KCl. The protein concentration in the cell-free extract was 0.95 mg/ml.

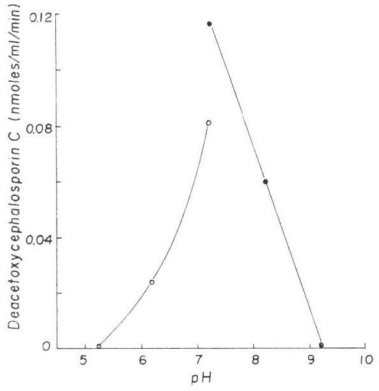


Fig. 5. Effect of temperature on ring-expansion activity.

Ascorbate, $FeSO_4$, and ATP were present at 0.67 mM, 0.04 mM, and 0.83 mM, respectively. PEP and PK were not present. The protein concentration in the cell-free extract was 0.64 mg/ml.

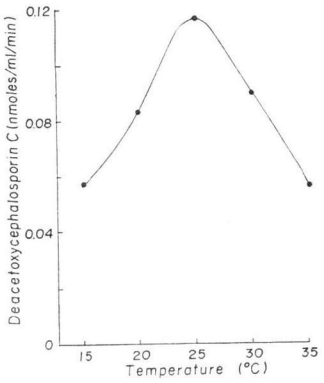


Fig. 6. Effect of penicillin N concentration on ring-expansion activity.

Ascorbate, $FeSO_4$, and ATP were present at 0.67 mM, 0.04 mM, and 0.83 mM, respectively. PEP and PK were not present. The protein concentration in the cell-free extract was 0.95 mg/ml. Penicillin N concentration is based on bioassay, not weight, because of its lability and lack of purity.

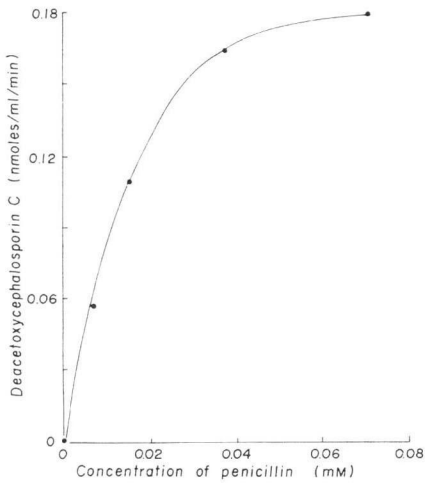
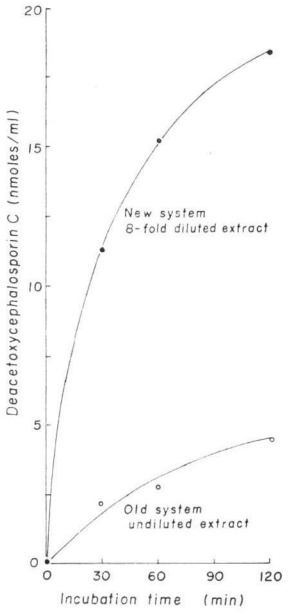


Fig. 7. Comparison of ring-expansion activity in the previous system[2] (containing 4.2 mM ATP, 8.3 mM PEP, and 83 μg/ml PK) with the new system (containing 0.67 mM ascorbate, 0.04 mM $FeSO_4$, and 0.83 mM ATP).

The cell-free extract contained 6.2 mg protein/ml. It was not diluted for use in the old system, but was diluted 8-fold for use in the new system.



ATP system, we subjected the product to bioautography. The cell-free extract used in this experiment was diluted in water instead of buffered salts (to lower the salts concentration) to facilitate chromatographic separation. The product behaved like deacetoxycephalosporin C (Table 1). Data es-

tablishing this compound as the product using labeled penicillin N has been submitted elsewhere (J. E. BALDWIN, P. D. SINGH, M. YOSHIDA, Y. SAWADA, and A. L. DEMAIN, submitted).

Comparison of Conversion Rates of Old and New systems

Fig. 7 compares the activities of the previous system[2] and the newly devised system. The 8-fold diluted cell-free extract in the new system converted penicillin N to deacetoxycephalosporin C at an initial rate more than 8 times faster than an undiluted extract in the old system. Thus the effective improvement factor is 60- ~ 70-fold.

Time of Harvest

The major difficulty in obtaining active cell-free preparations has involved choosing the proper harvest time. Although we had previously chosen 50 hours as the best time, this was not reproducible between fermentations. To compound the problem, ring-expansion activity disappeared soon after reaching its peak.

Table 1. Identification of the product by chromatography.

Material spotted	Rf value[a]	
	Paper chromatography[b]	Thin-layer chromatography[c]
Product	0.51	0.41
Deacetoxycephalosporin C	0.48	0.41
Cephalosporin C	0.44	0.35
Deacetoxycephalosporin C plus product	0.51	[d]
Cephalosporin C plus product	0.49, 0.44	[d]

a) Detected on plates seeded with *P. aeruginosa* Pss. Agar contained penicillinase.
b) Paper chromatography was done with the cell-free reaction mixture on Whatman No. 1 paper in the descending direction. The solvent system was acetonitrile - water (4: 1, v/v). The chamber was saturated with vapor from *n*-propanol - pyridine - acetic acid - acetonitrile - water (45: 30: 9: 40: 36, v/v).
c) TLC was done with the cell-free reaction mixture on cellulose plates (20×20 cm; Eastman Kodak Co.) in the ascending direction. The solvent system was butanol - acetic acid - water (3: 1: 1).
d) TLC was not done for these materials.

One way to improve reproducibility was to prepare and inoculate the sterile fermentation medium in the batch mode rather than in individual flasks. After inoculation, the seeded medium was dispensed into sterile flasks (see Materials and Methods) and incubated on the shaker.

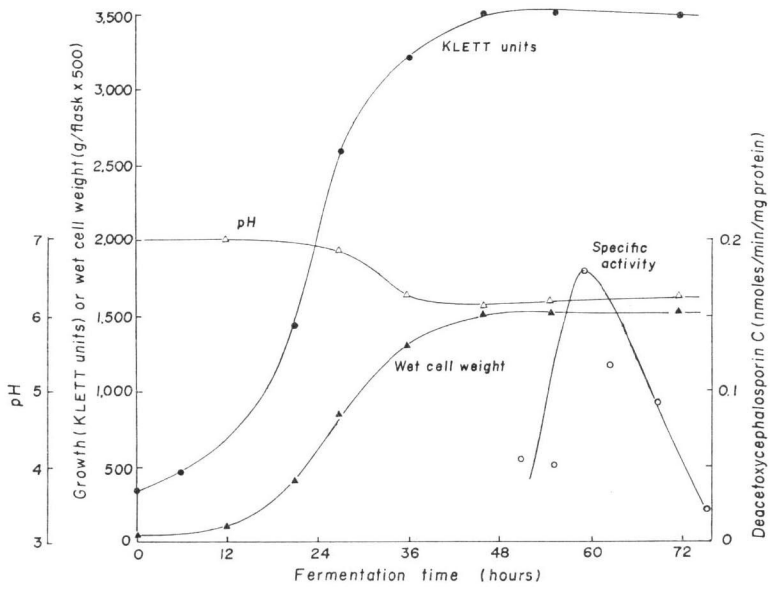
Another improvement was the development of the new cell-free reaction system. Under the previous system, usually only one harvest time point in the fermentation had activity; earlier and later samples were completely inactive. With the new system, even some of the early and late time points had usable activity.

A third improvement was the monitoring of growth in each fermentation by measuring culture absorbance. The absorbance correlated very well with cell mass and allowed us to follow growth with the colorimeter. Peak enzyme activity usually occurred 13 hours after growth ceased (Fig. 8). Thus, using this relationship and the new cell-free reaction procedure, we have been able to produce consistently cell-free extracts with activity. This has been confirmed by J. E. BALDWIN and P. D. SINGH at Oxford University (personal communication).

Despite the fact that ring-expansion activity is labile during the extended fermentation, activity is stable when culture flasks are merely refrigerated. In one experiment, cells were harvested, washed in 0.05 M McILVAINE buffer (pH 7.2), and divided into two portions. One portion was used immediately to prepare cell-free extracts and the other was stored at 4°C for 3 days before preparation of protoplast lysates. The specific activities of the two samples were virtually identical.

Fig. 8. Growth of *Cephalosporium acremonium* M-0198, pH change, and specific ring-expansion activity as functions of time.

The contents of triplicate flasks were pooled and analyzed for each time point.



Discussion

TURNER *et al.*[8] discovered that the dioxygenase, which catalyzes the oxidation of deacetoxycephalosporin C to deacetylcephalosporin C, was stimulated by $FeSO_4$, ascorbate, and α-KG; these factors were tested on the penicillin N ring-expansion reaction by HOOK *et al.*[5] We have confirmed the latter's positive results, including the finding that α-KG is not necessary for stimulation. Our studies revealed that $FeSO_4$ was optimal at 0.04 mM and ascorbate at 0.67 mM. In all of our experiments, these were freshly prepared.

We were surprised to find that in the presence of ascorbate and $FeSO_4$, our previously used concentrations of ATP, PEP, and PK (4.2 mM, 8.3 mM, and 80 μg/ml, respectively) were extremely inhibitory. A marked precipitation was associated with this inhibition when these energy factors were used in the presence of ascorbate and $FeSO_4$. The problem was solved by omitting PEP and PK, which we found were unnecessary. ATP, however, was stimulatory when used at 0.83 mM, 20% of its earlier concentration.

Despite the fact that our data indicated as early as 1976 that penicillin N was a precursor of a cephalosporin, other investigators have had a difficult time achieving the conversion in their laboratories. Although HOOK *et al.*[5] confirmed our results, we have been concerned about the low degree of conversion we had obtained. The problem involved several factors: (1) lack of the correct cofactors; (2) chemical lability and impurity of the substrate, penicillin N; (3) instability of the supersensitive assay organisms; (4) lack of predictability of the time of peak enzyme activity. The first problem was solved by replacing PEP and PK by ascorbate and $FeSO_4$ and decreasing the ATP concentration. Although the second problem remains, it is not a major one. The assay organism's instability has been circumvented by storing the culture in 50% glycerol at −65°C. Problem four, a major one, has been made less severe in three ways. First, we make up seeded medium in one batch rather than in individual flasks. Second, the presence of the cofactors in the assay allows us to pick up activity in certain cases even when the harvest time is earlier or later than desired, because of the 60- to 70-fold increase in rate of reaction. Third, we have used culture turbidity to follow growth; enzyme activity peaks about 13 hours after growth ceases. This latter result has markedly reduced the problem of harvest time selection.

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