Proc. Natl. Acad. Sci. USA
Vol. 75, No. 12, pp. 6253–6257, December 1978
Microbiology

Cell-free ring expansion of penicillin N to deacetoxycephalosporin C by *Cephalosporium acremonium* CW-19 and its mutants

(antibiotics/biosynthesis/cephalosporins/β-lactam/fermentation)

M. YOSHIDA*, T. KONOMI*, M. KOHSAKA*, J. E. BALDWIN†, S. HERCHEN†, P. SINGH†, N. A. HUNT*, AND A. L. DEMAIN*‡

*Department of Nutrition and Food Science and †Department of Chemistry, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

Communicated by Gerald N. Wogan, August 14, 1978

ABSTRACT **To examine microbiological ring expansion of penicillin N to a cephalosporin, we obtained five mutants of *Cephalosporium acremonium* blocked in β-lactam antibiotic biosynthesis from 2500 survivors of mutagenesis. In submerged fermentation, mutants M-0198, M-0199, and M-2351 produced no β-lactam antibiotic (type A), whereas mutants M-1443 and M-1836 formed penicillin N but not cephalosporin C (type B). Cell-free extracts of type A mutants converted penicillin N to a cephalosporin; those of type B mutants did not. The product of the cell-free reaction was identified as deacetoxycephalosporin C by thin-layer chromatography, paper chromatography, paper electrophoresis, and enzyme tests. These data strongly support our hypothesis that penicillin N is an intermediate of cephalosporin biosynthesis.**

Production of penicillin N and cephalosporins by *Cephalosporium acremonium* proceeds via the tripeptide δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (1, 2). The tripeptide is produced by a reaction involving L-α-aminoadipyl-L-cysteine and L-valine, during which the valine is converted to the D-form. The final steps of cephalosporin C production involve the hydroxylation of deacetoxycephalosporin C to deacetylcephalosporin C followed by acetylation of the latter to cephalosporin C (3). Virtually nothing is known about the steps leading from the tripeptide to penicillin N or deacetoxycephalosporin C, except that neither free 6-aminopenicillinic acid nor 7-aminocephalosporanic acid appears to be involved (4).

Of great interest has been the question of whether penicillin N and the cephalosporins are products of a branched pathway or whether the former is a precursor of the latter. The chemical transformation of a penicillin into a cephalosporin, achieved by Morin and co-workers (5), involves the activation of the sulfur atom by oxidation, cleavage of the carbon-sulfur bond by introduction of a double bond, and reclosure of the ring. Until our work (6), such ring expansion had never been observed in a biological system.

A major breakthrough was the development by Abraham's group (4, 7) of a cell-free system (using lysates of protoplasts of *C. acremonium*) capable of converting labeled valine or the tripeptide to a compound that appeared to be penicillin N. Bost and Demain (8) confirmed the conversion of labeled valine to a penicillin. Neither group, however, observed labeling of any cephalosporin. Kohsaka and Demain (6), using a cell-free system supplemented with penicillin N under conditions of high aeration, observed the formation of an antibiotic detectable by a strain of *Escherichia coli* supersensitive to β-lactam antibiotics. The antibiotic activity was destroyed by a broad-spectrum β-lactamase but not by a narrow-spectrum penicillinase. These properties are consistent with the possibility that the product was a cephalosporin. The present studies confirm and extend the results of Kohsaka and Demain (6) and give evidence in support of a microbial ring expansion of penicillin N to deacetoxycephalosporin C.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

MATERIALS AND METHODS

Unless stated otherwise, all media and conditions for growing *C. acremonium* CW-19 and its mutants were as described (9) and all experimental methods were as in our previous paper (6).

Natural penicillin N, deacetylcephalosporin C, and deacetoxycephalosporin C were gifts from R. L. Hamill (Lilly Research Laboratories, Indianapolis, IN). 6-Aminopenicillinic acid was obtained from C. Vezina and K. Singh of Ayerst Laboratories (Montreal, Canada).

Screening Method for Mutants Blocked in Cephalosporin Biosynthesis. Spores and mycelia of *C. acremonium* CW-19 were harvested from a slant with 5 ml of 0.05 M phosphate buffer (pH 7.0). The suspension was filtered through filter paper (no. 595, Schleicher and Schuell, Inc.), and the filtrate (containing mainly spores) was treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. Mutagenesis was carried out at 32°C for 60 min in a test tube containing 1.8 ml of spore suspension and 0.2 ml of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine solution containing 1.0 mg/ml in phosphate buffer. After mutagenesis, the spores were centrifuged at 1000 × *g* for 10 min and washed with 10 ml of phosphate buffer. Several dilutions were spread on plates of Czapek–Dox agar; these were incubated at 25°C for 7 days. The resultant colonies were transferred in a regular pattern to plates of Czapek–Dox agar and allowed to grow for 5 days.

Cylindrical plugs (diameter, 5 mm) were cut around the colonies with sterile plastic straws and incubated on assay plates seeded with the cephalosporin C-detection organism, *Alcaligenes faecalis* (ATCC 8750). Plugs without a zone of inhibition around them were considered to contain presumptive cephalosporin nonproducing mutants. These were then tested on two additional plates of Czapek–Dox agar seeded with one of the assay organisms supersensitive to β-lactam antibiotics; one of the plates contained penicillinase (Bacto-penase concentrate, 10^7 units/ml; Difco) (at the concentration used, 10,000 units/ml, this penicillinase attacks penicillin N but not the cephalosporins). Mutants whose plugs produced no inhibitory zones on either plate were designated type A; those that exhibited a zone only in the absence of penicillinase were designated type B. Mutants that produced zones against the supersensitive assay organism in the presence of penicillinase were considered to be weak producers of cephalosporins and were discarded. Both type A and B mutants were grown at 25°C in shaken 250-ml erlenmeyer flasks containing 40 ml of complex (10) or chemi-

‡ To whom reprint requests should be addressed at: Room 56-123, Massachusetts Institute of Technology, Cambridge, MA 02139.

cally defined (9) fermentation medium. Assays for penicillin N and cephalosporins were conducted as described below.

Preparation of Cell-Free Extract. Five milliliters of a 3- to 4-day-old seed culture was used to inoculate 40 ml of chemically defined medium in a 250-ml erlenmeyer flask. The culture was incubated at 25°C on a rotary shaker (5-cm-diameter orbit) at 250 rpm. The mycelia were harvested at various times during the fermentation. The flasks were divided into groups of six, and the contents of each group were pooled. The mycelia were washed twice with 60 ml of sterile distilled water and resuspended in a 250-ml erlenmeyer flask containing 40 ml of 0.05 M McIlvaine's buffer (pH 7.2) and 0.01 M dithiothreitol (Sigma). This suspension was incubated for 1 hr at 28°C with shaking at 150 rpm. The mycelia were filtered, washed twice with 40–50 ml of chilled sterile distilled water, and resuspended in a 250-ml erlenmeyer flask containing 40 ml of 0.05 M McIlvaine's buffer (pH 7.2) with 1.0 M NaCl, 0.02 M $MgSO_4$, 160 mg of *Cytophaga* lytic enzyme L_1 (Gallard-Schlesinger Chemicals, Carle Place, NY), and 160 mg of *Arthrobacter* zymolyase (Kirin Brewery, Tokyo, Japan). The suspension was incubated at 28°C for 3 hr with shaking at 120 rpm. The resulting protoplast suspension was centrifuged at 800 × *g* for 10 min. The pellet was washed three times with 60 ml of 0.05 M Tris buffer, pH 7.2/0.01 M KCl/0.01 M $MgSO_4$/1 M sucrose. After the third wash, the pellet was resuspended in 6 ml of 0.05 M Tris buffer, pH 7.2/0.01 M KCl/0.01 M $MgSO_4$ and placed in the freezer (−20°C) for 30–60 min. After thawing, the suspension was centrifuged at 3000 × *g* for 5 min. The supernatant fluid was the cell-free extract.

Cell-Free Reaction. To 1 ml of cell-free extract in a 10-ml erlenmeyer flask were added 10–30 μg of penicillin N activity, 5 μmol of adenosine triphosphate (Sigma), 10 μmol of phospho*enol*pyruvate (Sigma), and 100 μg of pyruvate kinase (Sigma). The final pH of the cell-free system was adjusted to 7.2. The mixture (1.2 ml total volume) was incubated for 3–5 hr at 25°C on a shaker at 250 rpm. *E. coli* Ess (6), E. coli Ess 22-31, or *Pseudomonas aeruginosa* Pss was used as assay organism. These β-lactam supersensitive mutant strains are about equally sensitive to cephalosporin C, deacetoxycephalosporin C, deacetylcephalosporin C, and penicillin N. Bioactivity was assayed by placing paper discs containing 25 μl of unknowns or standards on plates of antibiotic medium no. 5 (Difco) with and without 100,000 units of penicillinase (Difco) per 10 ml of agar seeded with the assay organism. After incubation at 37°C for 15 hr, zones of inhibition were measured. Cephalosporin C (obtained from C. A. Claridge, Bristol Laboratories, Syracuse, NY) was used as the standard. Any activity noted at zero time was subtracted from all subsequent assay values. Total β-lactam antibiotics were estimated on the plates without penicillinase. Cephalosporins were determined on the plates containing penicillinase. Subtraction of the cephalosporin value from total β-lactam antibiotic activity gave the penicillin N value.

Action of Enzymes on the Cell-Free Reaction Product Mixture. The reaction product mixture (125 μl) was exposed to 2000 units of penicillinase, to two concentrations (4 and 20 μl) of β-lactamase, or to a combination of 2000 units of penicillinase and 20 μl of β-lactamase. The β-lactamase was prepared from sonically ruptured cells of *Enterobacter cloacae* strain AAA 482 and was provided as a liquid preparation by L. Fare (Smith Kline & French Laboratories); this crude enzyme attacks not only the three cephalosporins but also penicillin N. Total volume in all cases was 147 μl. Incubation was static at 37°C for 5 hr. Samples were taken throughout the incubation, and residual activity was determined by the disc-agar diffusion assay (no penicillinase in the agar) with *P. aeruginosa* Pss as described above.

Purification of Product. Fifteen milliliters of the reaction mixture were treated with 75% (vol/vol) methanol to precipitate protein and other large molecules; the precipitates were removed by centrifugation at 3800 × *g* for 10 min. The supernatant fluid was evaporated to dryness under reduced pressure. Ten milliliters of water was added to the dried sample, and the pH was adjusted to 3–4 with H_2SO_4. Two grams of Amberlite XAD-2 polymeric adsorbant (gift of Smith Kline & French) was suspended in 50% (vol/vol) methanol and transferred to a glass column (0.5 × 30 cm); the column was successively washed with 1 M NaOH, 0.5 M H_2SO_4, and distilled water until the pH of the effluent was 3.0. The sample solution was applied to the column, which was then eluted with 10 ml of 10% (vol/vol) isopropyl alcohol; 2-ml fractions were collected. The fractions containing β-lactam antibiotics were detected by assay with *P. aeurginosa* Pss; those containing penicillin N or a particular cephalosporin were pooled and concentrated under reduced pressure to 1 ml.

Thin-Layer Chromatography. Thin-layer chromatography was carried out at room temperature on cellulose plates (20 × 20 cm; Eastman Kodak Co.) with the following solvent systems: (*i*) *n*-butanol/acetic acid/water, 3:1:1 (vol/vol); (*ii*) methanol/*n*-propanol/water, 6:2:1; (*iii*) *n*-propanol/water, 7:3. Double development in the same direction was used. The chromatograms were allowed to dry in air between the two developments.

Paper Chromatography. Chromatography was done with Whatman no. 1 chromatography paper in the descending direction. The developing solvent was acetonitrile/water, 80:20 (vol/vol) (11). The chromatography chamber was saturated with the vapor from *n*-propanol/pyridine/acetic acid/acetonitrile/water, 45:30:9:40:36 (vol/vol).

Paper Electrophoresis. Electrophoresis was performed on Whatman no. 1 paper (2 × 40 cm) at 40 V/cm for 60 min in 10% (vol/vol) acetic acid (pH 2.2) in a Gelman electrophoresis chamber.

Bioautography. Bioautograms were prepared by applying developed chromatograms to the surface of large agar plates seeded with one of the supersensitive assay organisms in the presence and absence of penicillinase. The plates were incubated overnight at 37°C.

RESULTS

Cell-Free Conversion by Parent Culture *C. acremonium* CW-19. As described in our preliminary communication (6), extracts of the parent culture produced an antibiotic activity that, unlike the substrate (penicillin N), was stable to penicillinase. Because the product(s) was destroyed by the broad-spectrum β-lactamase preparation from *E. cloacae*, we assumed it to be one or more cephalosporins. The dependence of the conversion on added penicillin N is shown in Fig. 1. The amount of cephalosporin synthesized was proportional to protein concentration between 3 and 7 mg/ml of reaction mixture (Fig. 2). Under normal experimental conditions, cell-free extracts with protein concentrations between 6 and 7 mg/ml were obtained. An energy-generating system (ATP, phospho*enol*pyruvate, and pyruvate kinase) was required for maximal cephalosporin production from penicillin N. Slight production of cephalosporin(s) was observed without the energy-generating system, but its addition resulted in a marked increase in the synthesis of cephalosporin(s).

The above studies were done with crude (about 15% pure) natural penicillin N. When synthetic penicillin became available, it was tested as a substrate. It was also converted to cephalosporin(s).

In the experiment shown in Fig. 3, production of cephalosporin(s) was linear for about 3 hr and reached its maximum

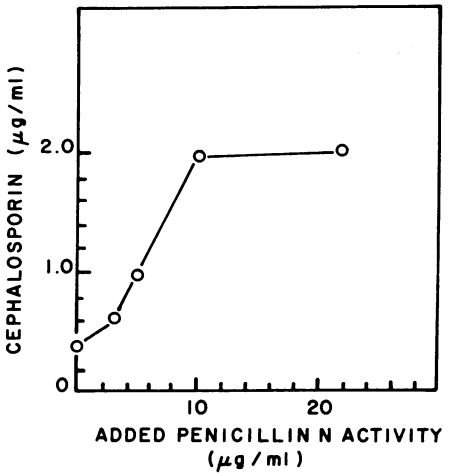

FIG. 1. Dependence of cephalosporin production by cell-free extracts of *C. acremonium* parent strain CW-19 on addition of penicillin N.

after about 5 hr. In this experiment (which was one of the most successful), approximately 80% of the penicillin N was converted to cephalosporin(s); however, the degree of conversion varied markedly from one cell-free preparation to another. Culture age strongly influenced the activity of the resulting cell-free extract; with strain CW-19, extracts prepared from 56-hr cultures grown in chemically defined medium were considerably more active than those from 44- or 68-hr cultures.

The activity of cell-free extracts was rather stable at −20°C. After 240 hr, 70% of the initial activity was observed.

Isolation of Mutants. Of a total of 2500 colonies tested, three mutants were of type A (producing neither detectable penicillin N nor a cephalosporin) and two were of type B (producing only penicillin N) (Table 1). All mutants were prototrophic.

Activity of Mutant Extracts. If our hypothesis (that penicillin N is an intermediate in cephalosporin biosynthesis) is correct, then type A mutants should be blocked before penicillin N, and their extracts should be able to convert penicillin N to cephalosporin(s). Fig. 4 shows that this was so with type A mutant M-0198. The two other type A mutants (M-0199 and M-2351) were also capable of converting penicillin N to cephalosporin(s). The activity of the M-0198 cell-free extract was completely lost after it was heated at 100°C for 5 min in 0.05 M Tris buffer (pH 7.2). Cycloheximide (100 μg/ml) did not inhibit the conversion.

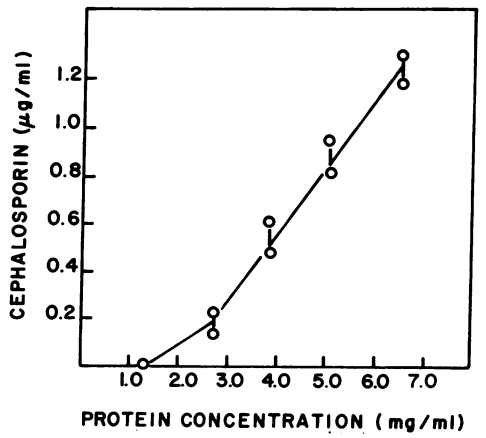

FIG. 2. Cephalosporin synthesis by cell-free extracts of *C. acremonium* parent strain CW-19 with various amounts of enzyme protein; 10 μg of penicillin N activity was added per ml of reaction mixture. Protein was determined by the procedure of Lowry *et al.* (12).

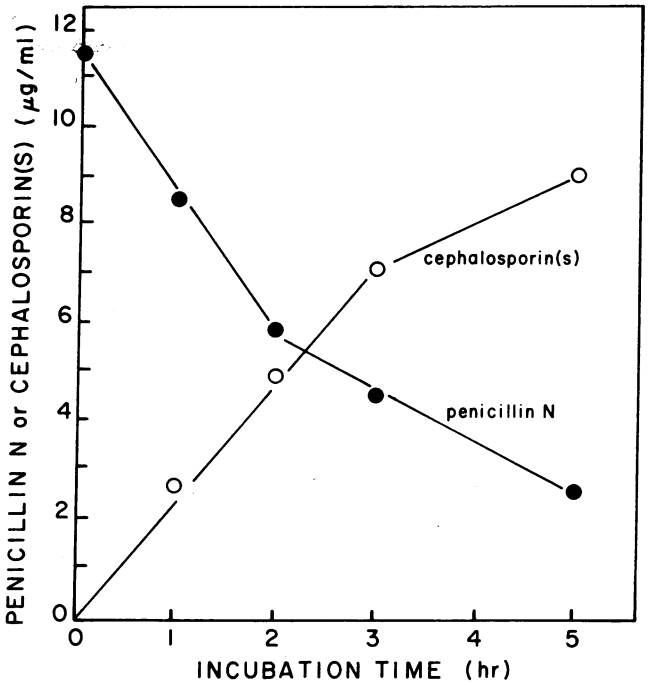

FIG. 3. Time course of conversion of penicillin N to cephalosporins by cell-free extracts of *C. acremonium* parent strain CW-19.

The optimal age of mycelia of mutant M-0198 for preparation of extracts was found to be 50 hr (Fig. 5).

Type B mutants were also tested in the cell-free conversion. Our hypothesis predicts that these cephalosporin-negative, penicillin N-positive cultures should be blocked in the penicillin N → cephalosporin conversion. Fig. 6 shows the inability of cell-free extracts of type B mutant M-1443 to effect the conversion. Extracts of M-1836, the other type B mutant, were similarly inactive.

Enzymatic Inactivation of Reaction Product Mixture. Fig. 7 shows the effect of penicillinase and two concentrations of *E. cloacae* β-lactamase on the unpurified reaction product mixture. Penicillinase and both concentrations of β-lactamase partially decreased the antibacterial activity of the reaction product mixture, indicating that the mixture contained residual penicillin N and cephalosporin(s). Also shown is the effect of these enzymes on penicillin N—i.e., complete destruction by penicillinase and partial destruction by the high concentration of β-lactamase but none by the low concentration. Deacetoxycephalosporin C was completely destroyed by either concentration of β-lactamase but not by penicillinase. When incubated with these same enzymes, the mixture of deacetoxycephalosporin C plus penicillin N behaved in a manner similar to that of the reaction product mixture.

Identification of Product. After partial purification, the

Table 1. Synthesis of β-lactam antibiotics by parent and mutant strains of *C. acremonium*

Culture		Penicillin N, μg/ml		Cephalosporins, μg/ml	
Mutant type	Number	Complex medium	Defined medium	Complex medium	Defined medium
Parent	CW-19	600–800	400–500	1300–1500	300–400
A	M-0198	<1	<1	<1	<1
A	M-0199	<1	<1	<1	<1
A	M-2351	<1	<1	<1	<1
B	M-1443	50–100	50–100	<1	<1
B	M-1836	50–100	50–100	<1	<1

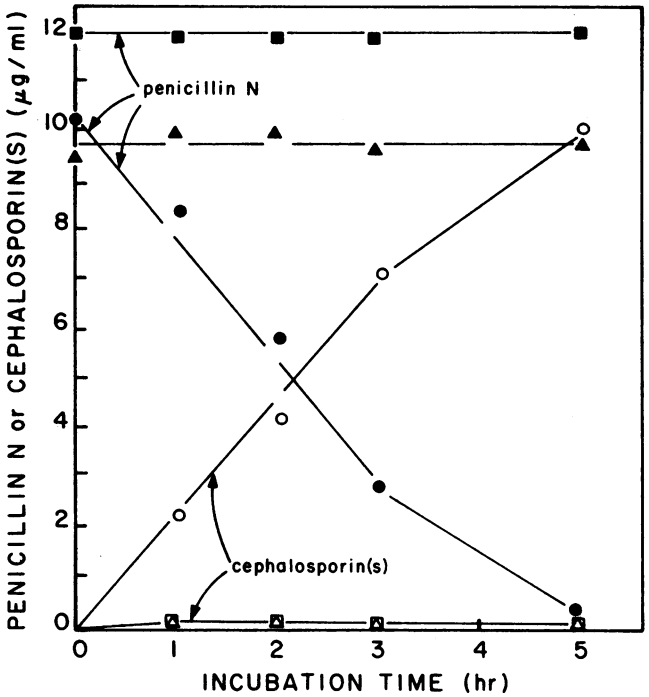

FIG. 4. Conversion of penicillin N to cephalosporin(s) by cell-free extract of *C. acremonium* type A mutant M-0198. ○, ●, Untreated extract; □, ■, boiled extract; △, ▲, no extract.

solution was chromatographed on thin-layer cellulose plates in three solvent systems and bioautographed. The purified product had a mobility similar to that of cephalosporin C and deacetoxycephalosporin C but different from that of penicillin N, deacetylcephalosporin C, and 6-aminopenicillinic acid (Table 2). As expected, none of the inhibitory zones of the cephalosporins was affected when the chromatograms were bioautographed on a penicillinase-containing plate, whereas that of penicillin N standard was eliminated.

To further establish the identity of the reaction product, paper chromatography in an acetonitrile/water system was

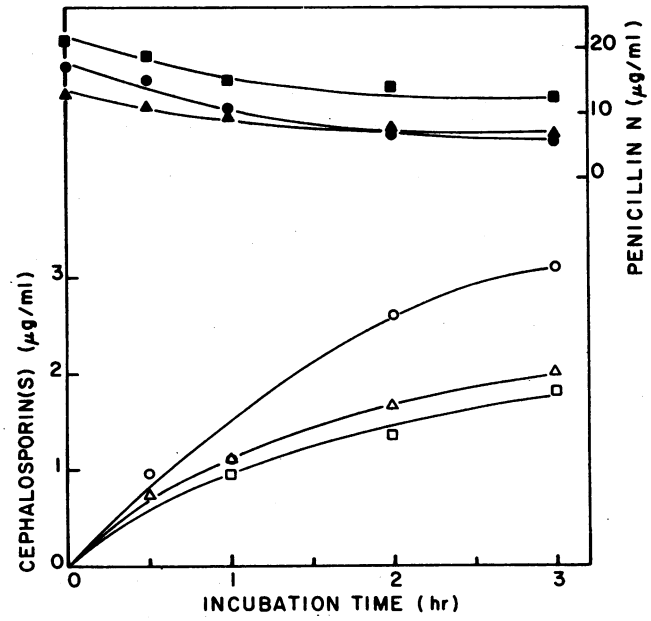

FIG. 5. Influence of culture age on conversion of penicillin N to cephalosporin(s) by cell-free extract of *C. acremonium* type A mutant M-0198. Ages of mycelia used to prepare extracts: ○, ●, 50 hr; △, ▲, 60 hr; □, ■, 70 hr.

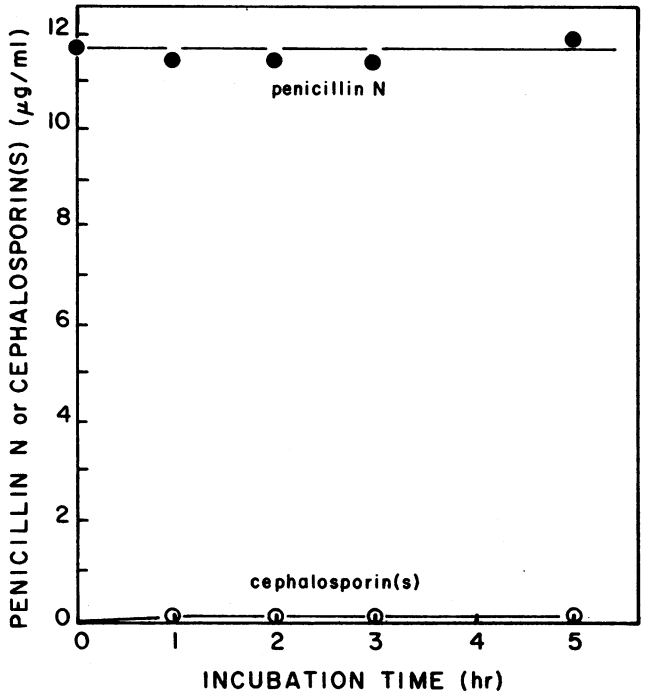

FIG. 6. Lack of conversion of penicillin N by cell-free extract of *C. acremonium* type B mutant M-1443.

conducted. The product and deacetoxycephalosporin C behaved in an identical manner, whereas cephalosporin C differed from both (Fig. 8 *upper*). The product also appeared identical to deacetoxycephalosporin C on paper electrophoresis (Fig. 8 *lower*).

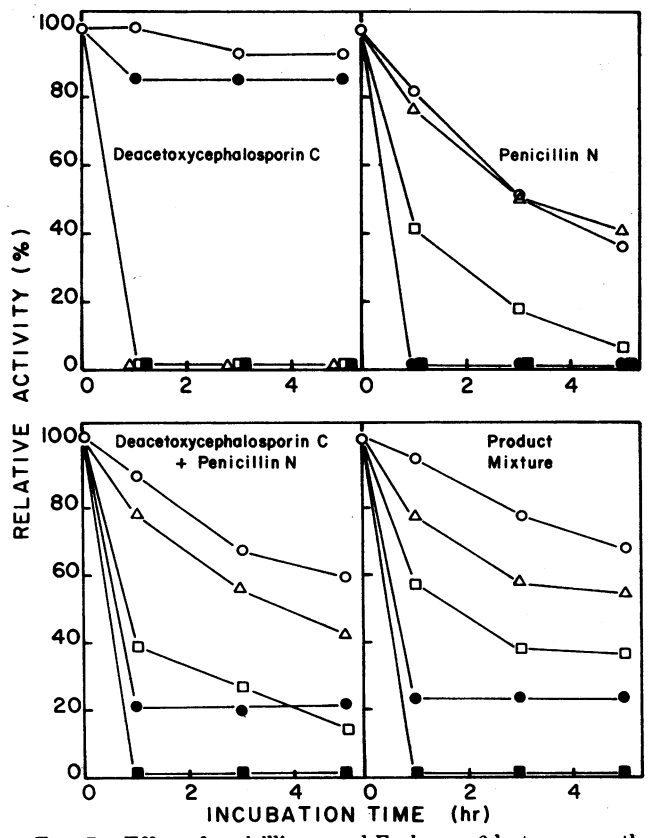

FIG. 7. Effect of penicillinase and *E. cloacae* β-lactamase on the antibiotic activity of the cell-free reaction product mixture. ○, No enzymes; ●, penicillinase; △, 4 μl of β-lactamase; □, 20 of μl β-lactamase; ■, penicillinase + 20 μl of β-lactamase.

Table 2. Thin-layer chromatography on cellulose of product and β-lactam antibiotics in three solvent systems

Material	System 1*	System 2†	System 3‡
Product	0.52	0.70	0.59
Penicillin N	0.69	0.79	0.65
Deacetoxycephalosporin C	0.53	0.68	0.59
Deacetylcephalosporin C	0.36	0.66	0.53
Cephalosporin C	0.50	0.70	0.60
6-Aminopenicillinic acid	—	—	0.70

* Butanol/acetic acid/water, 3:1:1.
† Methanol/*n*-propanol/water, 6:2:1.
‡ *n*-Propanol/water, 7:3.

DISCUSSION

A key question of β-lactam biosynthesis has been whether penicillin N is a precursor of the cephalosporins or whether these two types of antibiotics are distinct products of a branched pathway (Fig. 9). Our preliminary data (6) suggested the former hypothesis to be correct. The data obtained in the present study strengthen this conclusion by showing that type A mutants (which produce neither penicillin N nor cephalosporins in fermentation) carry out the cell-free conversion of penicillin N to cephalosporin(s), but type B mutants (which produce penicillin N but no cephalosporins in fermentation) do not. Further evidence includes the behavior of the cell-free reaction mixture upon exposure to the broad-spectrum β-lactamase of *E. cloacae* and to penicillinase. Furthermore, the R_F values of the purified product in three thin-layer chromatography systems, one paper chromatography system, and paper electrophoresis indicate that it is deacetoxycephalosporin C.

We recognize that further proof must be obtained to establish the linear pathway as the mechanism of biosynthesis of β-lactam antibiotics in *C. acremonium*. Conversion of labeled penicillin N and isolation of the reaction product will constitute proof of this hypothesis. At present, the hypothesis is consistent with our data and with the isolation of penicillin N-positive, cephalosporin-negative mutants and the lack of isolation of penicillin-negative, cephalosporin-positive mutants by previous workers (13, 14). It should be noted that our mutant-screening procedure would not have detected the latter type of mutant.

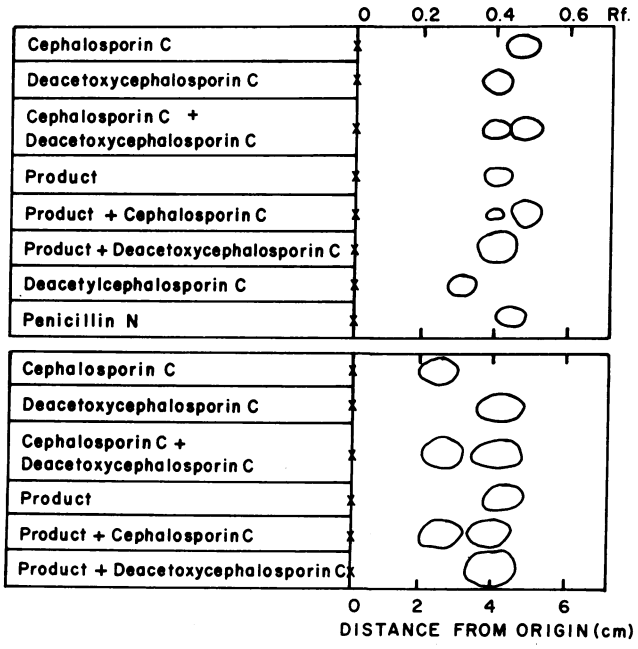

FIG. 8. Bioautography of purified product and β-lactam antibiotics. (*Upper*) After descending paper chromatography in acetonitrile/water, 80:20. The chamber was saturated with vapor of *n*-propanol/pyridine/acetic acid/acetonitrile/water, 45:30:9:40:36. Double development was used. (*Lower*) After paper electrophoresis at 40 V/cm for 60 min in 10% (vol/vol) acetic acid, pH 2.2.

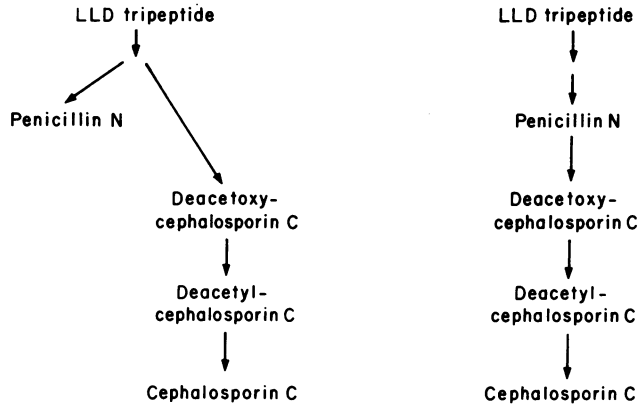

FIG. 9. Hypothetical branched and linear pathways of β-lactam antibiotic biosynthesis in *C. acremonium*.

Of practical significance for further work with the cell-free system is the absence of background antibiotic activity in extracts from type A mutants. The high background activity of the parental culture CW-19 complicates the interpretation of cell-free experiments.

We acknowledge the advice of C. H. Nash III and J. Valenta of the Smith Kline & French Laboratories on the isolation of cephalosporins. We thank R. L. Hamill, C. Vezina, K. Singh, C. A. Claridge, and L. Fare for various materials. We thank the Fujisawa Pharmaceutical Co. for the awarding of study leaves to M.K., T.K., and M.Y. The early part of this work by M.K. and A.L.D. was suported by Grant BMS 75-17527 from the National Science Foundation. The later experiments of T.K., M.Y., and A.L.D. were supported by a grant from the Industrial Division of the Bristol-Myers Company.

1. Loder, P. B. & Abraham, E. P. (1971) *Biochem. J.* **123**, 471–476.
2. Loder, P. B. & Abraham, E. P. (1971) *Biochem. J.* **123**, 477–482.
3. Fujisawa, Y., Kitano, K. & Kanzaki, T. (1975) *Agric. Biol. Chem.* **39**, 2049–2055.
4. Abraham, E. P. (1974) *Biosynthesis and Enzymatic Hydrolysis of Penicillins and Cephalosporins* (University of Tokyo Press, Tokyo).
5. Morin, R. B., Jackson, B. G., Mueller, R. A., Lavagnino, E. R., Scanlon, W. B. & Andrews, S. L. (1963) *J. Am. Chem. Soc.* **85**, 1896–1897.
6. Kohsaka, M. & Demain, A. L. (1976) *Biochem. Biophys. Res. Commun.* **70**, 465–473.
7. Fawcett, P. A., Usher, J. J., Huddleston, J. A., Bleaney, R. C., Nisbet, J. J. & Abraham, E. P. (1976) *Biochem. J.* **157**, 651–660.
8. Bost, E. P. & Demain, A. L. (1977) *Biochem. J.* **162**, 681–687.
9. Drew, S. W. & Demain, A. L. (1975) *Eur. J. Appl. Microbiol.* **2**, 121–128.
10. Caltrider, P. G. & Niss, H. G. (1966) *Appl. Microbiol.* **14**, 746–753.
11. Higgens, C. E., Hamill, R. L., Sands, T. H., Hoehn, M. M., Davis, N. E., Nagarajan, R. & Boeck, L. D. (1974) *J. Antibiot.* **27**, 298–300.
12. Lowry, O. H., Rosebrough, N. J., Farr, A. C. & Randall, R. S. (1951) *J. Biol. Chem.* **193**, 265–275.
13. Stauffer, J. F., Schwartz, L. J. & Brady, C. W. (1966) *Dev. Ind. Microbiol.* **7**, 104–113.
14. Lemke, P. A. & Nash, C. H. (1972) *Can. J. Microbiol.* **18**, 255–259.