Formation of Desacetylcephalosporin C in Cephalosporin C Fermentation

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The origin of desacetylcephalosporin C in cephalosporin C fermentation broths was investigated. Esterase activity was detected in cell-free extracts of *Cephalosporium acremonium*, but these extracts failed to deesterify cephalosporin C. When cephalosporin C was added to sterile and inoculated fermentation media, the antibiotic decayed at nearly identical rates. The formation of desacetylcephalosporin C during the fermentation was measured by quantitative chromatography and by the incorporation of valine-*1*-^{14}C into the molecule. The rate constants obtained from the results of these experiments were equivalent to those for the decay of cephalosporin C in sterile and inoculated media. The data demonstrate that desacetylcephalosporin C is produced by nonenzymatic hydrolysis of cephalosporin C.

Cephalosporin C, an antibacterial compound, was first isolated by Newton and Abraham (10). The structure and properties of this antibiotic were reported by Abraham and co-workers (1, 2, 3, 8, 10). Jeffery et al. (8) found that either enzymatic or alkaline hydrolysis of cephalosporin C produced a compound with reduced biological activity. This new compound, designated desacetylcephalosporin C, was structurally similar to cephalosporin C, except that the acetoxyl group was replaced with a hydroxyl function. Desacetylcephalosporin C has been detected in both intracellular and extracellular fluids of *Cephalosporium* sp. (11–13).

Early in our studies on the biosynthesis of cephalosporin C, desacetylcephalosporin C was consistently detected in *C. acremonium* culture filtrates. Because of the structural similarity of desacetylcephalosporin C to cephalosporin C, a study was undertaken to determine the origin of desacetylcephalosporin C and its relationship to cephalosporin C biosynthesis.

MATERIALS AND METHODS

The culture conditions and fermentation media used in this study have been previously described (5, 7). The concentration of cephalosporin C in culture filtrates was determined by the biological assay method of Demain and Newkirk (6), except that *Salmonella gallinarum* was used as the test organism. The identity of cephalosporin antibiotics was determined by bioautography. Culture filtrates were spotted on Whatman no. 1 filter paper and subsequently developed in a methanol–*n*-propanol–water (6:2:1, v/v) solvent system by descending chromatography. The R_F values of the cephalosporins were determined by placing the air-dried chromatogram on an agar plate seeded with *S. gallinarum* for 1 hr. The chromatograms were then removed, and the plates were incubated for an additional 18 hr at 25 C. Authentic cephalosporin compounds (or derivatives) were used as standards.

The concentration of desacetylcephalosporin C in culture filtrates was determined by streaking filtrates containing approximately 1 mg of cephalosporin C on a 9 × 10 inch (22.9 × 25.4 cm) piece of Whatman 3 MM filter paper. By use of the ascending technique, the sheets were chromatographed in 66% acetonitrile. The chromatograms were air-dried, and the position of antibiotic zones was located by ultraviolet light. The antibiotic zones were cut from the chromatogram and the antibiotic was eluted from the paper with water. After filtration, the optical densities of the eluates were determined at 260 mμ. Under these conditions, 1 μg of either cephalosporin C or desacetylcephalosporin C per ml produced an optical density of 0.0209.

Cell-free extracts of *C. acremonium* were prepared by disrupting mycelia in an Omni-mixer. A 10-g amount of wet cells was mixed with 30 ml of 0.05 M potassium phosphate buffer, *p*H 7.0, and 10 g of glass beads. After blending for 10 min at 5,000 rev/min and 4 C, the brei was centrifuged at 5,000 × *g* for 10 min. The pellet was discarded, and the esterase activity of the supernatant fluid was determined spectrophotometrically (9) with *p*-nitrophenylacetate as the substrate.

The incorporation of L-valine-*1*-^{14}C (Calbiochem, Los Angles, Calif.) into cephalosporin C and desacetylcephalosporin C was studied by adding 2 mc of the amino acid to the fermentation flasks at 48 hr. Unlabeled L-valine was added to give a final concentration of 3 mg/ml. After incubation for the required time, broth samples were filtered and chromatographed on Whatman no. 1 filter paper. The chroma-

tograms were developed with the upper phase of butan-1-ol–acetic acid–water (4:1:4, v/v). After development, the chromatograms were air-dried, and cephalosporin C and desacetylcephalosporin C were detected by ultraviolet-light absorption. The antibiotic zones were cut from the paper, placed in 15 ml of scintillation solvent (Packard Instrument Co., Downers Grove, Ill.) and counted in a Packard Tri-Carb liquid-scintillation counter.

RESULTS

The appearance of desacetylcephalosporin C in the fermentation broths is shown in Fig. 1. Although the method did not allow adequate quantitation, the concentration of the antibiotic appeared to increase with the age of the fermentation. Cephalosporin C and penicillin N were detected in the 72-hr sample, but desacetylcephalosporin C was not.

Since it was possible that desacetylcephalosporin C was formed by the action of an esterase on cephalosporin C, cell-free extracts were prepared and assayed for esterase activity. These extracts showed significant esterase activity with *p*-nitrophenylacetate as the substrate (Table 1). Like other esterases, the activity was inhibited by di-isopropylfluorophosphate and neostigmine, but not by potassium cyanide. Because the assay method did not lend itself to direct measurement
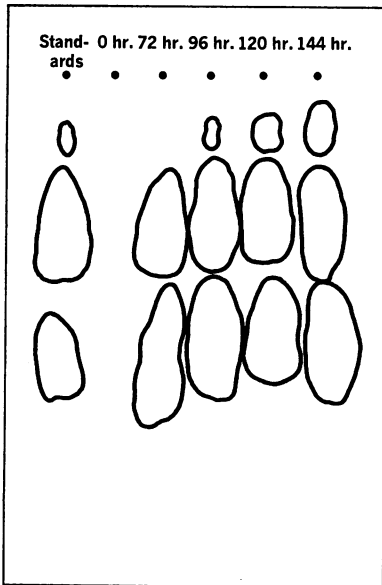
TABLE 1. *Effect of inhibitors on esterase activity in cell-free extracts of C. acremonium*

Inhibitor[a]	Optical density change/min	Percentage of control
None	0.186	100
Di-isopropylfluorophosphate (0.01 M)	0.090	48
Ethyl acetate (3%)	0.072	39
Neostigmine (0.001 M)	0.165	89
Potassium cyanide (0.001 M)	0.185	100
Cephalosporin C (0.005 M)	0.188	100

[a] Inhibitors were added to the complete reaction mixture.

of the hydrolysis of cephalosporin C, the action of the enzyme on the antibiotic was tested indirectly. If the extracts hydrolyzed cephalosporin C, this compound might be expected to inhibit competitively the activity of the enzyme on *p*-nitrophenylacetate. However, no reduction in esterase activity was observed when cephalosporin C was added to the reaction mixture. To eliminate the possibility that the enzyme was specific for *p*-nitrophenylacetate, ethylacetate was also tested as a competitive inhibitor. This ester decreased enzymatic activity approximately 61%.

Incubation of cephalosporin C under controlled conditions in sterile fermentation medium decreased the concentration of the antibiotic (Fig. 2). A decrease in the concentration of cephalosporin C was also observed when the compound was added to inoculated broth (Fig. 2). Cephalosporin C decreased in concentration at a similar rate in both systems. If it is assumed that the amount of cephalosporin C added to the broth at zero-time is equivalent to the concentration of cephalosporin C plus degradation products at any given time, then a rate constant for the degradation of cephalosporin C may be calculated according to the equation:

$$2.303 \log \quad x/(x - y) = kt$$

where x = the average total cephalosporin in fermentation broths at any given time or the amount of cephalosporin C added to the broth at zero-time, y = the degradation products of cephalosporin C measured at any given time, t = time in hours, and k = the rate constant in t^{-1}.

By use of the data presented in Fig. 2 and the above equation, the rate constants were calculated

FIG. 1. *Bioautographs of cephalosporin C fermentation broths sampled at the times indicated. A 40-μliter amount of filtered broth was applied to the paper at the indicated position. The standard zones represent, with increasing mobility, authentic desacetylcephalosporin C, cephalosporin C, and penicillin N.*

to be 0.0055 and 0.0054 for sterile and inoculated broth, respectively.

To study more critically the formation of desacetylcephalosporin C, L-valine-1-^{14}C was added to 48-hr fermentations. Broth samples were removed from the fermentation as indicated in Fig. 3, and the distribution of radioactivity in the antibiotics was determined. The broth samples were also assayed for cephalosporin C and desacetylcephalosporin C by the quantitative chromatography procedure. These data reveal that total cephalosporin synthesis (cephalosporin C + desacetylcephalosporin C) remained nearly linear and that the percentage of desacetylcephalosporin C increased with time. The rate constants were calculated from both radioactivity and quantitative chromatography data and found to be 0.0052 and 0.0056, respectively.

DISCUSSION

Theoretically, four possibilities exist for the production of desacetylcephalosporin C in fermentation broths: (i) the antibiotic is biosynthesized by a pathway distinct from that for the formation of cephalosporin C; (ii) desacetylcephalosporin C is produced by the enzymatic hydrolysis of cephalosporin C; (iii) the compound is formed by the nonenzymatic hydrolysis of cephalosporin C; or (iv) desacetylcephalosporin C is produced by a combination of any or all of
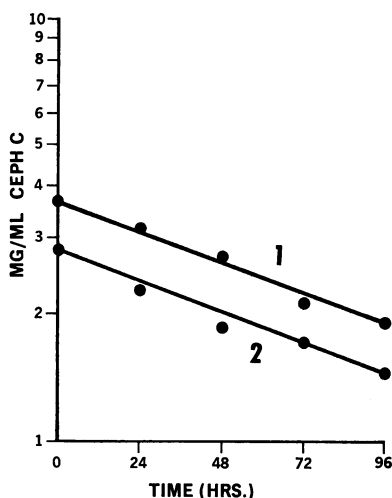
FIG. 3. *Incorporation of valine-1-^{14}C into cephalosporin C and desacetylcephalosporin C. Valine-l-^{14}C was added to 48-hr fermentations and incubation was continued for an additional 68 hr. Samples were withdrawn at the times indicated and processed as described in Materials and Methods. (1) Cephalosporin C plus desacetylcephalosporin C. (2) Cephalosporin C. (3) Disintegrations per minute (DPM) of cephalosporin C plus desacetylcephalosporin C. (4) DPM of cephalosporin C. (5) Desacetylcephalosporin C. (6) DPM of desacetylcephalosporin C.*

TABLE 2. *Comparison of theoretical and experimental values for the concentration of desacetylcephalosporin C in fermentation broths*

Time	Theoretical degradation[a] (A)	Observed degradation[a]	
		B	C
hr	*%*	*%*	*%*
0.0	0.0	0.0	0.0
16.0	4.2	3.4	—
24.0	6.2	6.8	5.0
41.5	10.2	8.8	—
44.5	10.9	8.4	12.3
64.5	14.9	15.0	—
67.5	15.5	16.3	17.8

[a] (A) Values based on data presented in Fig. 2, curve 1. (B) Values based on the incorporation of valine-1-^{14}C into cephalosporin C and desacetylcephalosporin C (Fig. 3). (C) Values based on results of quantitative chromatography measurements (Fig. 3).

FIG. 2. *Degradation of cephalosporin C in fermentation broth. (1) Cephalosporin C was added to sterile broth (pH 6.5 to 7.0) and treated as if the broth had been inoculated. Samples were withdrawn and assayed at times indicated. (2) Cephalosporin C was added to inoculated broth and assayed at the times indicated. Control fermentations were used to differentiate the amount of cephalosporin C added and that synthesized by the organism.*
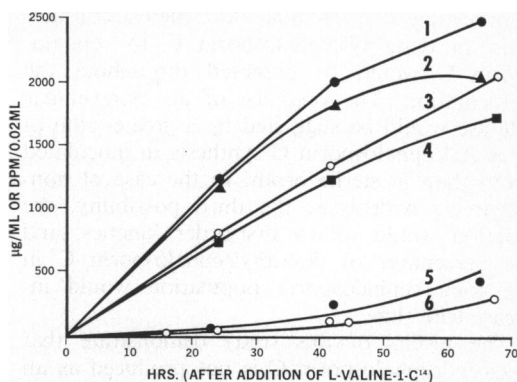
the foregoing possibilities. If possibility i were operative, the amount of desacetylcephalosporin C would not be related to the breakdown of cephalosporin C and its formation would not necessarily correspond to the synthesis of cephalosporin C. Enzymatic hydrolysis of cephalosporin C could occur either intra- or extracellularly. If desacetylcephalosporin C is formed intracellularly and it is assumed that its formation

is occurring at a maximal rate, then a constant ratio of desacetylcephalosporin C to cephalosporin C would be observed throughout the fermentation. The presence of an extracellular esterase would be suggested by a greater rate of desacetylcephalosporin C synthesis in inoculated broth than in sterile broth. In the case of nonenzymatic hydrolysis, the third possibility, the reaction would follow first-order kinetics, and the percentage of desacetylcephalosporin C in the total cephalosporin population would increase with time.

The results of this study demonstrate that desacetylcephalosporin C is not produced as an enzymatic de-esterification product of cephalosporin C. Cephalosporin C failed to inhibit competitively esterase activity with p-nitrophenylacetate as a substrate. Furthermore, no difference was observed in the rate constants when cephalosporin C was added to inoculated and uninoculated broth.

If desacetylcephalosporin C was formed biosynthetically, the rates of formation would not correlate with those predicted from the degradation of cephalosporin C in sterile broth. Theoretical values for the formation of desacetylcephalosporin C were calculated from the degradation of cephalosporin C in sterile broth (Table 2). These values agree well with the percentages of desacetylcephalosporin C obtained by both incorporation studies and quantitative chromatography measurements (Table 2). Thus, the data demonstrate that desacetylcephalosporin C is produced by nonenzymatic hydrolysis of cephalosporin C and is not produced biosynthetically.

LITERATURE CITED

1. Abraham, E. P., and G. G. F. Newton. 1956. Experiments on the degradation of cephalosporin C. Biochem. J. 62:658–665.

2. Abraham, E. P., and G. G. F. Newton. 1956. A comparison of the action of penicillinase on benzylpenicillin and cephalosporin N and the competitive inhibition of penicillinase by cephalosporin C. Biochem. J. 63:628–639.

3. Abraham, E. P., and G. G. F. Newton. 1961. The structure of cephalosporin C. Biochem. J. 79:377–383.

4. Abraham, E. P., G. G. F. Newton, and S. C. Warren. 1964. Studies on the biosynthesis of the cephalosporins. I.A.M. Symposia on Microbiology 6:79–96.

5. Caltrider, P. G. and H. F. Niss. 1966. Role of methionine in cephalosporin synthesis. Appl. Microbiol. 14:746–753.

6. Demain, A. L., and J. F. Newkirk. 1962. Biosynthesis of cephalosporin C. Appl. Microbiol. 10:321–325.

7. Huber, F. M., and M. O. Redstone. 1967. Changes in the fatty acids of Cephalosporium acremonium and its growth medium. Can. J. Microbiol. 13:333–334.

8. Jeffery, J. D'A, E. P. Abraham, and G. G. F. Newton. 1960. Further degradation product of cephalosporin C. Biochem. J. 75:216–223.

9. Keay, L., and E. M. Crook. 1965. Effect of metal ions on hog liver esterase. Arch. Biochem. Biophys. 111:626–634.

10. Newton, G. G. F., and E. P. Abraham. 1955. Cephalosporin C, a new antibiotic containing sulfur and D-α-amino adipic acid. Nature 175:548.

11. Smith, B., S. C. Warren, G. G. F. Newton, and E. P. Abraham. 1967. Biosynthesis of penicillin N and cephalosporin C Biochem. J. 103:877–890.

12. Warren, S. C., G. G. F. Newton, and E. P. Abraham. 1967. Use of α-aminoadipic acid for the biosynthesis of penicillin N and cephalosporin C by a Cephalosporium sp. Biochem. J. 103:891–901.

13. Warren, S. C., G. G. F. Newton, and E. P. Abraham. 1967. The role of valine in the biosynthesis of penicillin N and cephalosporin C by a Cephalosporium sp. Biochem. J. 103:902–962.