Studies on the Cell-Free Biosynthesis of β -Lactam Antibiotics

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Cell walls of *Cephalosporium acremonium* mycelia were lysed by enzyme preparations from either *Helix pomatia* (snail) digestive juice or *Cytophaga*. The yield of protoplasts depended on the lytic-enzyme preparation and the age of the culture, and it increased after the mycelia were pretreated with dithiothreitol. A cell-free preparation, obtained by osmotic lysis of protoplasts, synthesized labelled penicillin N from L-[14 C]valine. Approx. 0.03–0.06% of the amino acid was incorporated into penicillin N. Under conditions of penicillin N synthesis, the broken-protoplast preparation failed to produce significant amounts of cephalosporin C or its precursors, deacetylcephalosporin C and deacetoxycephalosporin C.

In spite of extensive efforts in many laboratories, the pathway for biosynthesis of β -lactam antibiotics has not yet been determined. One reason for this situation has been the lack of an effective cell-free system for synthesis of these important molecules. Without such a system, precursor-feeding studies are difficult to interpret because of permeability problems e.g. the lack of uptake of 6-aminopenicillanic acid and 7-aminocephalosporanic acid (Abraham *et al.*, 1965). Other complications include the hydrolysis of possible precursor peptides [such as the L- or D-forms of δ -(α -aminoadipyl)-L-cysteine] at the surface of the cell (Loder *et al.*, 1969).

In early attempts to produce a cell-free system, cells broken by ultrasonic treatment or by grinding with sand or alumina failed to synthesize a detectable amount of antibiotic (Abraham, 1974; Fawcett *et al.*, 1976a), but were able to produce the intermediate tripeptide (L- α -aminoadipyl-L-cysteinyl-D-valine) from L- α -aminoadipyl-L-cysteine and DL-valine (Loder & Abraham, 1971). These results suggested that the enzymes responsible for the biosynthesis of β -lactam antibiotics are part of an unstable complex, and that the structure of this complex is destroyed by drastic treatments. Abraham *et al.* (1965) reasoned that the conversion of cells into protoplasts followed by gentle lysis might preserve the integrity of the biosynthetic enzymes. In their preliminary work, Duncan & Newton (1970) noted the incorporation of L-[14 C]valine into penicillin N by a sonicated protoplast preparation from *Cephalosporium acremonium*. [14 C]Penicillin N was identified by conversion into [14 C]penicillaminic acid on oxida-

tion with performic acid. Abraham (1974) reported that incubation of lysed protoplasts with DL-[14 C]-valine, δ -(L- α -aminoadipyl)-L-cysteine, ATP and an energy-generating system yielded, after chemical oxidation, a radioactive substance which behaved like penicillaminic acid. The same lysed protoplast system could weakly incorporate L- α -aminoadipyl-L-cysteinyl-D-valine (labelled with 3 H in the methyl group of the valine residue) into penicillin N (Fawcett *et al.*, 1976a); incorporation into cephalosporin C was not reported.

Although the study of β -lactam biosynthesis by this cell-free system is only at a preliminary stage, the approach seems very promising. We thus thought that confirmation of the activity of the system was important. In contrast with the indirect measurement of incorporation into penicillin N used by Fawcett *et al.* (1976a), we hoped to purify penicillin N and count its radioactivity directly. We also considered it necessary to determine whether labelled valine is incorporated only into penicillin N or also into cephalosporin C and the intermediates deacetylcephalosporin C and deacetoxycephalosporin C.

One of the experimental problems encountered in the study of incorporation of a labelled amino acid into four different β -lactam compounds is the analysis of the reaction products in the crude reaction mixture. After β -lactam compounds are added as carriers, these antibiotics have to be isolated in pure form, as one would expect in a system of low efficiency. The direct use of paper chromatography or t.l.c. with crude extracts can lead to errors in assigning the label to a given compound. Multiple purification steps provide one way of avoiding this difficulty, although they decrease recovery yields. The stability of β -lactam antibiotics is of utmost importance in the

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separation process, since penicillin N, cephalosporin C, deacetoxycephalosporin C and deacetylcephalosporin C have one or more sites susceptible to attack by H^+ or OH^- ions and water. At pH 2.5 and room temperature, cephalosporin C remains stable, whereas penicillin N is degraded to its penicilloic acid. Thus speed of analysis is important in the determination of precursor incorporation into β -lactam antibiotics.

In the present study, we used a purification scheme which combined column chromatography on charcoal, high-pressure liquid chromatography, t.l.c. and electrophoresis to separate the products and confirm cell-free incorporation of labelled valine into penicillin N.

Materials and Methods

Cultures

C. acremonium strain CW19 and the method for maintaining it have been described (Drew & Demain, 1975). The culture produces 400 μ g each of cephalosporin C and penicillin N/ml in the defined medium described by Drew & Demain (1975). *Alcaligenes faecalis* (A.T.C.C. 8750) was used for the determination of cephalosporin C, and *Sarcina lutea* (A.T.C.C. 9341) was the test organism for the determination of penicillin N.

Media

The seed medium and the chemically defined production medium described by Drew & Demain (1975) were used. The production medium contained 3.0 g of DL-methionine/litre, which served as the positive effector of antibiotic production. Conditions for seed development and fermentation in flasks were as previously described (Drew & Demain, 1975). The seed and production cultures were regularly checked by microscopic examination for bacterial contamination. Production flasks were removed at different times to measure cephalosporin C, penicillin N or dry cell weight, and to harvest mycelia for protoplast preparation.

Antibiotic determination

The bioassay organism for the cephalosporin C determination, *A. faecalis* A.T.C.C. 8750, is resistant to penicillin N and 'cephalosporin' P. The details of the agar-diffusion assay have been presented (Drew & Demain, 1975). The bioassay organism for the penicillin N determination, *S. lutea* A.T.C.C. 9341, is relatively insensitive to cephalosporin C and 'cephalosporin' P. This organism was grown to a cell density of 200–300 Klett units (approx. 1 g dry weight/litre) in a 250 ml flask containing 40 ml of heart infusion broth, and 10 ml of this suspension was used to inoculate 1 litre of heart infusion agar. Then 12 ml of the seeded agar was added to the plates,

which were kept at 0°C unless they were used immediately. Filtered broths were diluted 10-fold for the penicillin N assay in order to eliminate any significant interference by the steroidal 'cephalosporin' P. The agar-diffusion assay was carried out in a manner similar to that for cephalosporin C.

Dry cell-weight determination

Dry cell weight was determined as previously described (Drew & Demain, 1975).

Protein determination

The protein content of cell-free lysates or enzyme reaction mixtures was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

Protoplast formation

Experiments on protoplast formation were modelled on the procedure of Fawcett *et al.* (1973). The culture was grown in the production medium for periods of 48–90 h. Each 40 ml sample of broth was centrifuged for 20 min at 800g, after which the supernatant fluid was discarded. The mycelial pellet was washed three times in 20 ml of double-distilled sterile water and then harvested by centrifugation. The mycelia were resuspended in 0.1 M McIlvaine's citrate/phosphate buffer at pH 7.3 (McIlvaine, 1921), in the presence or absence of 0.01 M-dithiothreitol, and the suspension was incubated for 1 h at 28°C on a rotary shaker (200 rev./min). The suspension was centrifuged for 10 min at 800g, and the mycelia were washed twice with 25 ml of double-distilled sterile water. The mycelial pellet was resuspended in 10 ml of osmotic-stabilizing solution [McIlvaine's citrate/phosphate buffer (pH 6.8) with 0.8 M-NaCl and 0.02 M-MgSO₄] and various concentrations of the following lytic-enzyme preparations: *Helix pomatia* (snail) digestive juice, HPJ Special, Glusulase, chitinase, or dried lytic-enzyme preparation L₁ from *Cytophaga*. The suspension was incubated at 30°C on a rotary shaker at 120 rev./min for 3–4 h. Protoplast formation was determined microscopically by counting with a Levy-Hausser Counting Chamber (A-2906 Colorless Glass, area 0.0025 mm², depth 0.1 mm). The protoplasts and the remaining mycelia were centrifuged at 300g for 10 min (0°C) and the supernatant fluid was removed. The pellets were resuspended twice in the osmotic-stabilizing solution and centrifuged each time at 800g for 10 min at 0°C.

Separation of protoplasts from mycelia

The pellets containing protoplasts and residual mycelia were resuspended in 25 ml of the osmotic-stabilizing solution and passed through a packed glass-wool filter to eliminate any remaining clumps of mycelia. The mycelia retained by the filter were washed with the osmotic-stabilizing solution. Filtrates

and washes were combined and centrifuged for 20 min at 250g to eliminate the last bits of mycelia found on the bottom layer; the protoplasts remained in the supernatant fluid. The protoplasts were finally harvested by centrifugation at 5000g for 10 min. At each step of the procedure, the suspensions were examined microscopically.

Lysis of protoplasts

The protoplast pellet was resuspended in 1 ml of double-distilled sterile water, frozen for 20 min at -5°C , and allowed to thaw after 1 ml of 0.05M-Tris/HCl buffer (pH 7.0), containing 0.01M-MgSO₄ and 0.01M-KCl, was added. Lysis of protoplasts was checked by microscopic examination. We also determined the protein content of the resulting solution.

Synthesis of β -lactam antibiotics by cell-free lysates

The ability of protoplast lysates to synthesize penicillin N or cephalosporins was determined by

adding the following compounds to 1 ml of the crude lysate: 5 μmol of ATP, 9 μmol of phosphoenolpyruvate, 200 μg of pyruvate kinase, 5 μmol of L- α -amino adipic acid, 5 μmol of L-cysteine, 1 μCi of L-[U- ^{14}C]valine (specific radioactivity 200 $\mu\text{Ci}/\mu\text{mol}$). The mixture was incubated for 2 h at 28°C and then frozen at -20°C until it was analysed.

Analytical procedure for determination of L-[^{14}C]valine incorporation

We treated 1 ml of reaction mixture with 75% (v/v) methanol to precipitate protein and other large molecules, and then removed them by filtration through a 1.2 μm Millipore filter. Methanol was removed from the filtrate by evaporation *in vacuo* to dryness after 1 mg each of penicillin N and the three cephalosporins had been added as carriers.

We used column chromatography on granular charcoal (Abraham & Newton, 1954) as the first step in purification in order to adsorb 'cephalosporin' P

Fig. 1. High-pressure liquid chromatography of authentic compounds on a Sepcoate pellicular anion-exchange resin column (60.96 cm $\times$ 2.3 mm)

Ammonium formate buffer (0.1 M) pH 6.0 was used at a flow rate of 0.4 ml/min (2 MPa, 300 lb/in²). (a) Cephalosporin C; (b) deacetoxycephalosporin C; (c) deacetoxycephalosporin C + deacetylcephalosporin C + cephalosporin C + L-valine; (d) valine + penicillin N.

and nucleic acid-type impurities. Granulated charcoal (40–120 mesh; 2g) was suspended in water and transferred to a glass column (0.4cm×10cm). Then 10ml of 1M-HCl was percolated through the column, which was subsequently washed with water and 0.1M-potassium phosphate buffer, pH 6.0, until the pH of the eluate reached 6.0. The column was washed again with water (20ml). Water (2ml) was added to the dried sample and the solution was applied to the column, which was then eluted with 2ml of water followed by 10ml of 60% (v/v) acetone; fractions (0.1ml) were collected. The fractions containing cephalosporins and penicillin N were detected by assay with *A. faecalis* and *S. lutea* respectively. The fractions containing antibiotics were pooled and concentrated *in vacuo* to 0.1ml at 20–22°C.

The second part of the purification scheme involved high-pressure liquid chromatography on anion-exchange resin, which removed most of the labelled valine. Cation-exchange resins were also studied, but the β -lactam antibiotics were not sufficiently retained to allow even partial purification. Concentrated antibiotic solution (60 μ l) was injected into a column (length 60.96cm, internal diameter 2.3mm) filled with Sepcoate 7AN pellicular anion-exchanger (Separation Technology, Cambridge, MA, U.S.A.). Ammonium formate buffer (0.1M, pH 6.2) was passed through the column at 0.4ml/min under pressure of 2 MPa (300lb/in²), by using a high-pressure liquid-chromatography pump (Waters Associates, Milford, MA, U.S.A.). Cephalosporin derivatives were detected in the fractions with a u.v. recorder at 254nm; a refractive-index recorder was used to detect penicillin N and valine. Fractions suspected of containing antibiotics were examined further by bioassay. The retention times of penicillin N, deacetoxycephalosporin C, deacetylcephalosporin C and cephalosporin C standards ranged from 20 to 28 min (Fig. 1); valine was eluted earlier (approx. 10min). Fractions containing the β -lactam compounds were pooled and concentrated *in vacuo* to 100 μ l.

In the next step we used chromatography on thin-

layer cellulose plates to separate the antibiotics from each other. Cellulose has excellent partitioning properties, and cephalosporins and penicillins are relatively stable in the presence of this sorbent. R_F values of β -lactam antibiotics are shown in Table 1. The positions of the cephalosporins and penicillin N on the plates were detected on guide strips with ninhydrin reagent and by bioautography (Murakawa *et al.*, 1970) with *A. faecalis* or *S. lutea*. All the cephalosporins inhibit *A. faecalis*, but deacetylcephalosporin C is the only one active on the penicillin N assay organism, *S. lutea*. Purified extract (10 μ l) was chromatographed on a thin-layer cellulose plate (Eastman Kodak, Rochester, NY, U.S.A.) and developed with methanol/propanol/water (6:2:1, by vol.) in the first dimension. For further separation of the antibiotics, electrophoresis was carried out in the second dimension for 100 min at 25 V/cm. Owing to the instability of penicillin N, one of two solvent systems was used, depending on the aim of the experiment. To obtain a clear separation of the three cephalosporins from each other and from penicillin N, electrophoresis was carried out at pH 2.2 with 10% (v/v) acetic acid. Penicillin N is degraded under these conditions. To obtain undegraded penicillin N clearly separated from the cephalosporins, electrophoresis was performed in 0.05M-acetic acid, adjusted to pH 4.5 with pyridine. This latter system was not used to obtain isolated spots of the cephalosporins, since cephalosporin C and deacetoxycephalosporin C migrated at very similar rates.

When radioactivity was found in the penicillin N spot, we used penicillinase to confirm that the activity was indeed in penicillin N. Purified extract (10 μ l) was treated with 1 μ l of concentrated penicillinase to convert penicillin N into its penicilloic acid. The reaction mixture was chromatographed in butanol/acetic acid/water (4:1:5, by vol.) in the first dimension, and electrophoresis was then performed in the second dimension in the acetate/pyridine buffer, pH 4.5.

All spots corresponding to penicillin N, its penicilloic acid, or the three cephalosporins were scraped from the plates after detection by u.v. or ninhydrin.

Table 1. R_F values of penicillin N, deacetoxycephalosporin C, deacetylcephalosporin C and cephalosporin C determined by t.l.c. on cellulose

For details see the Materials and Methods section.

Antibiotic	Solvent system ...	R_F value	
		Butanol/acetic acid/water [4:1:5, by vol. (upper (organic) layer)]	Methanol/propanol/water (6:2:1, by vol.)
Penicillin N		0.62	0.47
Deacetoxycephalosporin C		0.50	0.30
Deacetylcephalosporin C		0.30	0.20
Cephalosporin C		0.44	0.36

Their radioactivities were measured in a Scintiverse mixture by liquid-scintillation counting.

Mineral salts solution

The salts solution contained the following, per litre: K_2HPO_4 , 1.1 g; KH_2PO_4 , 1.2 g; $Na_2SO_4 \cdot 10H_2O$, 12.6 g; $MgSO_4 \cdot 7H_2O$, 2.7 g; $CaCl_2 \cdot 2H_2O$, 0.55 g; $ZnSO_4 \cdot 7H_2O$, 0.2 g; $MnSO_4 \cdot H_2O$, 0.2 g; $CuSO_4 \cdot 5H_2O$, 0.05 g.

Chemicals

Cephalosporin C was a gift from Dr. C. A. Claridge of Bristol Laboratories (Syracuse, NY, U.S.A.). Deacetoxycephalosporin C, deacetylcephalosporin C and penicillin N were given by Dr. R. L. Hamill of Lilly Research Laboratories (Indianapolis, IN, U.S.A.). Additional penicillin N samples were gifts from Dr. J. Nüesch of CIBA-Geigy (Basel, Switzerland) and Dr. M. A. Pisano of John's University (Jamaica, NY, U.S.A.). L-[U- ^{14}C]-Valine (specific radioactivity 200 $\mu Ci/\mu mol$, was from ICN Pharmaceuticals, (Cleveland, OH, U.S.A.) Dithiothreitol, ATP, phosphoenolpyruvate and pyruvate kinase were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). L- α -Aminoadipic acid was from Calbiochem (San Diego, CA, U.S.A.). Lytic-enzyme preparations from *H. pomatia* digestive juice, β -glucuronidase, sulphatase and HPJ Special (stabilized with merthiolate) were from l'Industrie Biologique Francaise (Genevilliers, Seine, France). The lytic enzyme preparation glusulase was a gift from Endo Laboratories (Garden City, NY, U.S.A.). Fungal chitinase was from Schwarz/Mann (Orangeburg, NY, U.S.A.), and a lytic-enzyme preparation (L_1) from *Cytophaga* was first obtained gratis from Dr. D. J. D. Hockenull of Glaxo Laboratories (Ulverston, Lancs., U.K.) and later purchased from BDH Biochemicals (Poole, Dorset, U.K.). Penicillinase (Bactopenase), heart infusion broth and heart infusion agar were from Difco Laboratories (Detroit, MI, U.S.A.). We obtained 2,3,5-triphenyl-2H-tetrazolium chloride from J. T. Baker Chemical Co. (Phillipsburg, NJ, U.S.A.). Nutrisoy Flour 200 D was given by Archer Daniels Midland Co. (Decatur, IL,

U.S.A.), and Vita-Pro 80 was a gift from Rath Packing Co. (Waterloo, IA, U.S.A.). Hyamine hydroxide was a product of New England Nuclear (Boston, MA, U.S.A.), and Scintiverse was from Fisher Scientific Co. (Pittsburgh, PA, U.S.A.). β -Lactamase preparation from sonically ruptured cells of *Enterobacter cloacae* was a gift from Dr. L. Fare of Smith, Kline and French Laboratories (Philadelphia, PA, U.S.A.). This preparation hydrolysed both penicillin N and the cephalosporins.

Results

Formation of protoplasts

Since the present work was carried out with a strain of *C. acremonium* different from that used by Fawcett *et al.* (1973) in their protoplast studies, we needed to assess the effect of certain variables on protoplast formation. The culture used in the present work, strain CW19, is capable of greater cephalosporin C production than the strain used by Fawcett *et al.* (1973).

Under conditions similar to those of Fawcett *et al.* (1973), we found that the best conversion of cells into protoplasts occurred with 48–60h-old mycelia (Table 2) when the *Cytophaga* lytic-enzyme preparation was used. The suitability of a particular enzyme preparation depended on the age of the culture. For example, although *Cytophaga* lytic enzyme and *Helix* digestive juice had almost the same activity on 72h-old mycelia, the *Cytophaga* preparation was more active on 48 or 60h-old mycelia. These results indicate that the structure of the multilayered hyphal wall changes with the age of the mycelium.

Fawcett *et al.* (1973) showed that pretreatment of the mycelium with a thiol-group-specific reagent, which reduces disulphide bonds in protein-mannan complexes, enhances the production of protoplasts. We found that pretreatment with dithiothreitol also stimulated protoplast production in our strain of *C. acremonium*. Omitting dithiothreitol from the incubation mixture decreased protoplast counts by 25–50% with the various enzyme preparations.

The rate and extent of protoplast formation from 60h-old mycelia depended on the particular lytic enzyme preparation used. Fig. 2 shows the superiority of *Cytophaga* lytic enzyme L_1 over some of the other preparations. A concentration of 4mg/ml was suitable for good protoplast formation.

Microscopic examination showed that protoplasts were released at the growing hyphal wall behind the tip. With the *Cytophaga* lytic enzyme or the HPJ Special preparation, the whole cell wall appeared to be digested rapidly, leaving the protoplasts free in the osmotic-stabilizer. The protoplasts of *C. acremonium* CW19 are spherical and tend to agglutinate at high concentration (Plate 1). Even with a NaCl stabilizer, they swelled during incubation, doubling or trebling

Table 2. Effect of the age of the *Cephalosporium acremonium* culture on the conversion of mycelia into protoplasts
Damp-dry mycelia (2g) were incubated in a total volume of 10ml for 3h with *Cytophaga* lytic-enzyme preparation L_1 (10mg/ml) at 30°C.

Age of mycelia (h)	$10^{-7} \times$ No. of protoplasts/ml
48	6.5
60	7.5
72	2.0
90	1.0

in diameter. The average diameter of a protoplast was about 5 μ m. Protoplasts were osmotically fragile and started to burst when the NaCl concentration was lowered to 0.3M. One or two large vacuoles could be observed.

The purity of the protoplast suspension is important in the preparation of a cell-free lysate. Filtration under gravity through a sintered-glass funnel with a

large pore size (Fawcett *et al.*, 1973) significantly decreased the number of protoplasts recovered. An alternative procedure, consisting of filtration through glass-wool followed by centrifugation (described in the Materials and Methods section), was more satisfactory. The absence of residual mycelia was verified microscopically (Plate 1).

Cell-free synthesis of β -lactam antibiotics with L-[14 C]-valine as labelled precursor

The system used in our experiments (see the Materials and Methods section) was used by Duncan & Newton (1970) and Fawcett *et al.* (1976a). This system includes ATP, phosphoenolpyruvate, pyruvate kinase, labelled precursor, constituent amino acids and lysed protoplasts. The energy-generating system provides a sufficient supply of ATP, which is presumably necessary for the activation of amino acids, the formation of the intermediate tripeptide, and other steps in biosynthesis.

After the cell-free reaction mixture was incubated for 2 h at 28°C, the products were purified in a multi-step process, which included chromatography on granular charcoal, high-pressure liquid chromatography on an anion-exchanger, t.l.c. on cellulose and electrophoresis. With osmotically lysed protoplasts, incorporation of L-[14 C]valine (1 μ Ci/ml, specific radioactivity 10 μ Ci/ μ mol) into penicillin N was observed (Table 3); the degree of incorporation increased from 0.03 to 0.06% as we increased the amount of lysate. Despite the low conversions, the radioactivity in the reaction products could be unambiguously attributed to penicillin N. Further treatment of the purified product with penicillinase, followed by electrophoresis at pH 7.0, shifted the

Fig. 2. Protoplast formation by various lytic-enzyme preparations

Damp-dry mycelia (2mg) were incubated in 10ml of McIlvaine's citrate/phosphate buffer (pH 6.7) with 0.8M-NaCl and 0.02M-MgSO₄. The preparations and the protein content used were *Helix* digestive juice at 25mg/ml (∇) and 50mg/ml (Δ); HPJ Special at 15mg/ml (Δ) and 30mg/ml (∇); glusulase at 10mg/ml ($\circ$) and 20mg/ml ($\bullet$); *Cytophaga* lytic preparation L₁ at 4mg/ml ($\square$) and 8mg/ml ($\blacksquare$).

Table 3. Incorporation of [U- 14 C]valine into penicillin N

Reaction mixture (1 ml) contained 1 μ Ci of [14 C]valine, 5 μ mol of ATP, 10 μ mol of phosphoenolpyruvate and 400 μ g of pyruvate kinase in 0.05M-Tris/HCl buffer, pH 7.0. Radioactivity values are those of the corresponding spots, minus the radioactivity at the same position in a control experiment in which the cell-free lysate was boiled before being added to the reaction mixture. Data are averages for several experiments. —, No significant radioactivity was detected above the background. No radioactivity was incorporated into deacetoxycephalosporin C, deacetylcephalosporin C and cephalosporin C.

Concentration of cell-free lysate (ml/ml of reaction mixture)	Radioactivity (c.p.m.) incorporated into		Incorporation into penicillin N (%)
	Penicillin N	Penicilloic acid*	
0.1	—	—	0
0.2	—	—	0
0.3	150	—	0.030
0.4	325	250	0.061
0.5	350	350	0.062

* Gain of radioactivity in the position of penicilloic acid after treatment of the reaction mixture by penicillinase (2000 units of Bactopenase per 10 μ l of reaction mixture).

EXPLANATION OF PLATE I

Protoplasts of C. acremonium CW19 in McIlvaine's citrate/phosphate buffer (pH 6.8) containing 0.8M-NaCl+0.02M-MgSO₄ after treatment with Cytophaga lytic enzyme L₁ (4mg/ml) for 3 h at 30°C followed by removal of mycelia
(a) Protoplasts; (b) agglomerate of protoplasts after 150 min.

radioactivity from penicillin N to the corresponding penicilloate; the loss of radioactivity from penicillin N corresponded to the gain in that of penicilloic acid. No significant labelling of deacetoxycephalosporin C, deacetylcephalosporin C or cephalosporin C occurred. Occasionally we saw some radioactivity associated with one of the cephalosporins. However, the radioactive material showed no change in chromatographic or electrophoretic mobility after treatment with *E. cloacae* β -lactamase and thus could not have been caused by a labelled cephalosporin.

Discussion

One objective of our work was to confirm the activity of the cell-free system for biosynthesis of β -lactam antibiotics (Abraham, 1974). The findings of this group from Oxford indicated that gently lysed protoplasts of *C. acremonium* can serve as a broken-cell system in which the various steps of β -lactam biosynthesis could be studied. Earlier studies had demonstrated that β -lactam antibiotics are not synthesized by cell-free preparations made by more vigorous disruption techniques.

The reaction conditions in our cell-free experiments (buffer, pH, temperature, energy-generating system) were not optimized; they were the same as those used by earlier workers (Duncan & Newton, 1970; Abraham, 1974; Fawcett *et al.*, 1976a). In spite of the low efficiency of the cell-free system with L-[14 C]valine as a precursor, the labelling was unambiguously attributed to penicillin N and the estimated incorporation varied from 0.03 to 0.06%. These data are consistent with the results obtained by Duncan & Newton (1970) with their sonicated protoplast system. They showed a 0.032% conversion of [14 C]valine into penicillin N. Abraham's group (Abraham, 1974; Fawcett *et al.*, 1976a,b) observed 0.3% incorporation of the tripeptide L- α -amino-adipyl-L-cysteinyl-D-valine into penicillin N, by cell-free lysates. The lack of detectable incorporation of DL-[4,4- 3 H]valine into penicillin N observed by Fawcett *et al.* (1976b) was due to the use of a low concentration of valine of a low specific radioactivity (E. P. Abraham, personal communication). This was an intentional manipulation, since the amino acid was used as control for following the more intensive incorporation of intact 3 H-labelled tripeptides into penicillin N. Our failure to detect labelled cephalosporins in these experiments cannot be explained by degradation of these compounds during the purification procedure. Possible explanations could be (a) an unusually low extent of labelling, (b) instability of the enzyme responsible for cephalo-

sporin biosynthesis, or (c) absence of the proper conditions for conversion to cephalosporins.

In subsequent studies, we have obtained an improved cell-free preparation (Kohsaka & Demain, 1976), which converted penicillin N into cephalosporin only when subjected to a high degree of oxygen transfer. Since the present experiments were done without agitation or aeration, the lack of sufficient oxygen was probably responsible for the failure to incorporate label from valine into the cephalosporins.

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