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Biosynthesis of Penicillin N and Cephalosporin C

ANTIBIOTIC PRODUCTION AND OTHER FEATURES OF THE METABOLISM OF A *CEPHALOSPORIUM* SP.

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1. The production of penicillin N and cephalosporin C by two mutants of a *Cephalosporium* sp. has been studied with cultures grown in a chemically defined medium and with suspensions of washed mycelium in water or a buffered salt solution. 2. Antibiotic synthesis began at an early stage of growth and its rate per unit weight of mycelium appeared to pass its maximum as morphological changes were occurring in young hyphae. This rate subsequently declined, but rapid production could continue after net growth had ceased. 3. In a series of shake-flask fermentations in the growth medium, increases in the yield of penicillin N above the mean were correlated with much smaller increases in the yield of cephalosporin C and vice versa. 4. In suspensions of washed mycelium, moderate decreases in the efficiency of aeration increased the yield of penicillin N and decreased that of cephalosporin C. A similar result normally followed the addition of methionine to the suspension fluid, and in both cases there was usually an increase in the yield of the two antibiotics combined. 5. The apparent intracellular concentrations of the antibiotics were much lower than those attained extracellularly and also much lower than those of most of the amino acids in the intracellular pool. No detectable amount of [^{14}C]penicillin N added to the extracellular fluid was found to enter the mycelium. 6. Very small amounts of peptide material whose behaviour was similar to that of the sulphonic acid of δ-(α-aminoadipoyl)cysteinylvaline on paper electrophoresis at pH 1·8 were found in extracts of the mycelium that had been oxidized with performic acid. 6-Aminopenicillanic acid and 7-aminocephalosporanic acid were not detected. 7. Ultrasonic treatment of the mycelium resulted in rapid fragmentation of mycelial chains, rupture of many individual cells, and the liberation of amino acids and other substances into the medium. 8. Ultrasonically treated preparations synthesized penicillin N and cephalosporin C rapidly after a lag of 12hr. Antibiotic synthesis was accompanied by the growth of hyphae from swollen mycelial fragments and by the re-establishment of permeability barriers resulting in the uptake of amino acids from the medium.

Cephalosporium sp. C.M.I. 49137 and mutants obtained from this species produce both penicillin N and cephalosporin C, which are δ-(D-α-aminoadipoyl) derivatives of 6-aminopencillanic acid and 7-aminocephalosporanic acid respectively.

Experiments in which ^{14}C-labelled DL-amino acids were added to a mutant (8650) of this *Cephalosporium* sp. growing in a complex medium in shake flasks showed that α-aminoadipic acid, cysteine and valine were incorporated into side chain, β-lactam ring and dihydrothiazine ring respectively of cephalosporin C (Trown, Smith & Abraham, 1963). Further experiments indicated that the δ-(D-α-aminoadipoyl) side chain of this antibiotic is formed from acetate and α-oxoglutarate and that the acetoxy group is derived from acetate (Trown, Abraham, Newton, Hale & Miller, 1962; Trown, Sharp & Abraham, 1963).

Chemically defined media have been described for the growth of the *Cephalosporium* sp. and the formation of penicillin N and cephalosporin C, and the production of both antibiotics has been shown to be stimulated by D- or DL-methionine (Ott, Godzesky, Pavey, Farran & Horton, 1962; Miller, Kelly & Newton, 1956; Kavanagh, Tunin & Wild, 1958; Demain, Newkirk & Hendlin, 1963). Meth-

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ionine could be replaced by its structural analogue, D-norleucine, as a stimulator of cephalosporin C production, but no increase in the yield of this antibiotic was obtained by the addition of D- or DL-α-aminoadipic acid, L-valine or L-lysine to the culture medium and only slight stimulation was observed with L-cysteine (Demain *et al.* 1963).

Penicillin N and cephalosporin C have also been produced by washed mycelium of the *Cephalosporium* sp. suspended in phosphate buffer (Ott *et al.* 1962), or in a mineral salt solution buffered with phosphate (Demain, 1963*a*,*b*). Under the latter conditions L-[1-^{14}C]valine was incorporated efficiently into cephalosporin C (Demain, 1963*b*).

Demain (1963*a*) studied the behaviour of preparations obtained by ultrasonic treatment of a further mutant (C-91) of *Cephalosporium* sp. 8650 in a mineral salt solution buffered with phosphate. He reported that these preparations of broken cells synthesized cephalosporin C after a lag of 3hr. but that after centrifugation the supernatant alone produced none and the sediment alone very little.

With a view to obtaining more information about the incorporation of radioactive precursors into penicillin N and cephalosporin C, further studies were made of the production of these antibiotics by intact and broken mycelium respectively and of the concentrations of possible precursors in intracellular pools. The results of some of these studies are given in this paper.

METHODS

Assay of penicillin N and cephalosporin C. The antibiotics were assayed by the hole-plate method (Brownlee *et al.* 1948). The combined activity of penicillin N and cephalosporin C in solutions containing both substances was determined with *Salmonella typhosa* (strain Mrs S; Felix & Pitt, 1935) as test organism. This organism is ten times as sensitive to penicillin N as to cephalosporin C. Hence with mixtures containing a substantial proportion of penicillin N the zones of inhibition are virtually determined by the concentration of the latter. The concentration of cephalosporin C was determined with *Alcaligenes faecalis* A.T.C.C. 8750 (Claridge & Johnson, 1962) in agar containing penicillinase (Neutrapen; Burroughs Wellcome and Co., London). This strain of *Alcaligenes faecalis* is about ten times as sensitive to cephalosporin C as to penicillin N. The amount of penicillinase used (10000 units/l.; 3mg./l.) was 50 times that required to inactivate the penicillin in a solution containing 80 units/ml. under the conditions used. The activity of cephalosporin C was not affected by this concentration of the penicillinase.

Since only small amounts of penicillin N were available, this antibiotic, as well as cephalosporin C, was assayed against a standard preparation of cephalosporin C. Pure sodium cephalosporin C dihydrate is assumed to have an activity of 10 units/mg. A solution containing 0·05mg. of a preparation of penicillin N sodium salt/ml. (80% pure according to chemical assay by the hydroxamic acid method of Boxer & Everett, 1949) gave a zone of inhibition on plates seeded with *Salm. typhosa* with the same diameter as that produced by 4 units of cephalosporin C/ml. Pure penicillin N sodium salt was therefore assumed to have an activity of 100 units/mg. A solution containing both penicillin N and cephalosporin C whose assay was x units/ml. against *Salm. typhosa* and y units/ml. (corresponding to $100y\mu$g. of cephalosporin C/ml.) against *Alcaligenes faecalis* was therefore assumed to contain $x-y$ units/ml. ($10[x-y]\mu$g./ml.) of penicillin N.

Standard solutions containing 4, 2 and 1 units/ml. respectively were used in assays with *Salm. typhosa* and solutions containing 1, 0·5 and 0·25 unit/ml. respectively in assays with *A. faecalis*. The slopes of straight lines obtained from plots of log concentration of penicillin N against diameter of inhibition zone on plates seeded with *Salm. typhosa* were slightly less steep, in most cases, than the corresponding slopes with cephalosporin C. The ratio of the two slopes (p) varied from 0·7 to 1·0 and the lines intersected at 4 cephalosporin C units/ml. The true concentration, t units/ml., of an unknown solution of penicillin would thus be related to its apparent concentration, x units/ml., measured on the cephalosporin C standard line, by the expression:

$$\log t = p \log x + (1-p) \log 4$$

The difference between t and x diminishes as p approaches 1 or as x approaches 4. In the present work penicillin N was usually assayed at concentrations that gave activities between 2 and 6 units/ml. Under these conditions the maximum errors to be expected from the use of cephalosporin C instead of penicillin N, as a standard (with p 0·7) were −19·3% ($x=2$) and +12·8% ($x=6$) respectively. No attempt was made to eliminate these errors by determinations of p for different assays.

Cephalosporin P, in addition to penicillin N and cephalosporin C, was present in some of the culture fluids studied (Burton & Abraham, 1951). At the concentrations in which it was encountered, however, cephalosporin P was inactive against *Salm. typhosa* and *A. faecalis*.

Fungi used for antibiotic production. Cephalosporium sp. C.M.I. 49137 was isolated by Brotzu (1948). *Cephalosporium* sp. C.M.I. 49137, mutant 8650, was isolated by Mr R. Codner at the Medical Research Council's Antibiotics Research Station, Clevedon, Somerset. *Cephalosporium* sp., strain C-91 (MF-4239), was kindly provided by Dr A. L. Demain (Merck and Co. Inc., Rahway, N.J., U.S.A.). It was obtained from mutant 8650 and is unable to utilize nitrate as a source of nitrogen for growth.

Production of antibiotics in growth medium. (*a*) Preparations of spore suspensions and inocula. These were prepared from *Cephalosporium* spp. as described by Trown *et al.* (1962) except that the initial incubation of cultures of mutant 8650 on half-strength Czapek–Dox agar was for 7 days only and the initial growth of mutant C-91 was on Oxoid nutrient agar no. 2 for 7 days.

(*b*) Cultures in shaken flasks. Each flask (Erlenmeyer; normally 500ml.) had four vertical indentations, placed symmetrically, which were 6–7cm. long, 10mm. deep at the base and tapered to 3mm. in depth at the top. For maximum yields of cephalosporin C under the conditions employed it was necessary for the indentations to begin as near as possible to the base of the flasks.

Each flask contained 100ml. of sterile medium, 0·1ml. of oleic acid and 10ml. of inoculum. The flasks were placed

on a rotary shaker (140 rev./min. with a 2in. throw), which was surrounded by a box, kept at 27·5°, through which a slow stream of air was drawn. In some cases fermentations were carried out in 250ml. flasks with similar indentations, each flask containing 50ml. of medium.

The medium used had the same composition as the chemically defined medium no. 3 described by Demain *et al.* (1963) except that L-asparagine (7·5g./l.) was added in place of $(NH_4)_2SO_4$ (Abraham, Newton & Warren, 1964).

Production of antibiotics by suspensions of washed mycelium. (*a*) By intact mycelium. Cultures of the *Cephalosporium* sp. were harvested when antibiotic production had reached about half its maximum (usually 72hr. after inoculation) and the mycelium was separated by filtration through hardened filter paper (Green's Hyduro, 904) in a sterile Buchner funnel covered with a Petri dish. The mycelium was washed by three successive resuspensions in the funnel in 100ml. of sterile water and refiltration. Usually about 10g. of damp-dry mycelium was obtained per 500ml. flask.

The mycelium was resuspended in distilled water or in a buffered salt solution (6ml./g. of damp-dry cells). The salt solution was that described by Demain (1963*a*). Samples of the suspension were dispensed through a wide-mouthed pipette into sterile Erlenmeyer flasks with four indentations (usually 5ml. of 10ml. samples in 50ml. and 100ml. flasks respectively). The flasks were shaken on a rotary shaker (2in. throw) at 200 rev./min. and at 27·5°.

(*b*) By homogenized mycelium. In some cases a suspension of the mycelium in water or buffered salt solution (3ml./g.) was homogenized in a Potter–Elvehjem homogenizer cooled in ice (10 strokes with a Teflon pestle) and then diluted with an equal volume of water or buffered salt solution before incubation in shake flasks.

(*c*) By mycelium subjected to ultrasonic treatment. Homogenates prepared as previously described were diluted with an equal volume of water or buffered salt solution and samples (20ml.) of the resulting mixture were subjected to ultrasonic treatment in a jacketed glass vessel cooled with a stream of ethanol at 0°. An MSE ultrasonic disintegrator (60w, 20kcyc./sec.) was used with a 1cm. diam. titanium probe whose end was about 2mm. below the surface of the suspension. Treatment was usually continued for 30min.

The protein content of ultrasonic extracts of mycelium (supernatants obtained after centrifugation for 20min. at 16000*g*) was determined by the method of Lowry, Rosebrough, Farr & Randall (1951).

(*d*) By mycelium disrupted by ultrasonic treatment and shaking with glass beads. In some cases mycelium which had been subjected to ultrasonic treatment was disrupted further by shaking with glass beads (4mm. diam.) under the conditions described by Demain (1963*a*).

Effect of ultrasonic treatment on mycelial viability and morphology. Samples of suspensions of washed mycelium (before ultrasonic treatment, after treatment for varying periods of time and after subsequent resuspension in shake flasks) were diluted appropriately in water. Samples (0·5ml.) of the diluted suspensions were spread on Oxoid nutrient agar plates and colonies counted after 48 and 72hr. incubation at 27°. Dilutions were such that the number of colonies per plate was usually between 50 and 200. Samples at lower dilutions were also examined microscopically under phase-contrast or dark-ground illumination.

Fractionation of mycelium. (*a*) Estimation of intracellular penicillin N and cephalosporin C. Washed damp-dry mycelium was stirred with ice-cold 70% (v/v) ethanol (5ml./g.) and the suspension kept for 5hr. at 0°. The mycelium was filtered and washed with 0·3vol. of 70% ethanol. The combined filtrate and washing was concentrated *in vacuo* (to about 0·5ml./g. of original damp-dry mycelium) and the concentrate assayed for penicillin N and cephalosporin C.

(*b*) Intracellular amino acid pool. Two procedures were used for the extraction of intracellular amino acids and other substances. (1) Washed damp-dry mycelium was extracted with ice-cold 70% (v/v) ethanol (3ml./g. for 5min. The mycelium was filtered and extracted with ice-cold water (3ml./g.) for 5min., filtered again and washed on the filter with water (1ml./g.). The ethanol and water extracts were combined and freeze-dried after additional water had been added to reduce the ethanol content of the extracts to 10% (v/v). The weights of these dried extracts increased from 50mg./g. of damp-dry mycelium for cultures harvested at 48hr. to 70mg./g. for cultures harvested at 120hr. (2) Washed mycelium (5g.) from about 50ml. of culture fluid was extracted with hot 70% (v/v) ethanol (30ml.) as described by Arnstein & Morris (1960) in work with *Penicillium chrysogenum*. The weights of the dried extracts were similar to those obtained by procedure (1).

The quantity and nature of the amino acids extracted by these two procedures were very similar and also very similar to those in extracts of the mycelium with 5% (w/v) trichloroacetic acid at room temperature.

(*c*) Hot-trichloroacetic acid extracts. After extraction of the intracellular pool the filtered mycelium was treated with 5% (w/v) trichloroacetic acid (4ml./g.) at 90° for 15min. The cooled extract was adjusted to pH1 by addition of 6N-HCl and most of the trichloroacetic acid removed by three extractions with an equal volume of ether.

(*d*) Hydrolysis of mycelial protein. The solid remaining after extraction with trichloroacetic acid was kept for 30min. at 90° in 0·2N-NaOH (10ml./g.), separated by filtration and washed with 0·5vol. of water. The filtrate was neutralized with 2N-HCl and evaporated. The residue was hydrolysed in 6N-HCl (10ml./g.) at 105° for 16hr. The hydrolysate was concentrated and cooled and crystals of NaCl were removed by filtration. The filtrate was evaporated to dryness.

(*e*) Hydrolysis of mycelial residue. The solid remaining after extraction with 0·2N-NaOH was mixed with 6N-HCl and the mixture heated at 105° for 16hr. The hydrolysate was evaporated to dryness.

Amino acid analysis. The amino acids in the different mycelial extracts and hydrolysates were adsorbed from aqueous solution on columns (10cm. × 1cm. diam.) of Dowex (X4; H^+ form; 200–400 mesh) and the columns washed with water (30ml.). Amino acids were then eluted with N-NH_3 (30ml.) and the eluate was freeze-dried. Samples (10mg.) of the residues were dissolved in a 1·5% solution of performic acid in 98% (w/w) formic acid (2ml.), the solutions kept at −10° for 6hr. and then diluted with water (20ml.) and freeze-dried. The residues were analysed with an amino acid analyser (made by Arthur Guinness, Son and Co. Ltd.) similar to the one described by Moore & Stein (1951).

Paper chromatography and electrophoresis. Paper chrom-

atograms were run in butan-1-ol–acetic acid–water (4:1:4, by vol.) on Whatman no. 1 paper for 16hr. or with the same solvents (4:1:5, by vol.) on Whatman no. 4 paper for 4–6hr. at 4°. Electrophoresis was carried out on Whatman no. 1 or no. 4 paper: (1) at 70v/cm. in an apparatus similar to that of Katz, Dreyer & Anfinsen (1959) in pyridine acetate buffer (0·05M to acetate), pH4·5, or in 20% (v/v) acetic acid containing 2% formic acid, pH1·8; (2) at 13v/cm. in collidine acetate (0·05M to acetate), pH7·0, in an apparatus similar to that described by Flynn & de Mayo (1951). Amino acids and peptides were coloured with ninhydrin. Cephalosporin C and deacetylcephalosporin C were detected by their absorption of ultraviolet light (Abraham & Newton, 1961). Penicillin N and cephalosporin C were also detected by bioautography on plates seeded with *Salm. typhosa*.

Amino acid oxidases. Dried venom from *Crotalus adamanteus* (from Ross Allen's Reptile Institute, Silver Springs, Fla., U.S.A., supplied by Koch–Light Laboratories Ltd., Colnbrook, Bucks.) was used as a source of L-amino acid oxidase (Ratner, 1955; Wellner & Meister, 1961). The dried venom was dissolved in 0·2M-*N*-ethylmorpholine–HCl buffer, pH7·2, to give a concentration 10–12mg./ml. The solution was dialysed in Visking tubing at 4° for 4hr. against at least 50vol. of the same buffer. The pH of the dialysed enzyme was adjusted to 7·8 by addition of *N*-ethylmorpholine immediately before the enzyme was used (for pH–substrate relationships of L-amino acid oxidases see Paik & Kim, 1965). Oxidation of L-amino acids was measured manometrically in a Warburg apparatus at 38°, with air as the gas phase and 2N-KOH in the centre cup. The main compartment contained dialysed enzyme in *N*-ethylmorpholine–HCl buffer, pH8·5 (1ml.), catalase (5μl. of a suspension of a crystalline preparation from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany) and 0·1M-KCl (0·6ml.). The substrate, in water (0·2ml.), was placed in one side arm and appropriate L-amino acid in water (0·2ml.) in the other. The enzyme preparation used catalysed the oxidation of L-α-aminoadipic acid, but not that of L-aspartic acid, L-glutamic acid or L-proline.

D-Amino acid oxidase from hog kidney was from British Drug Houses Ltd., Poole, Dorset. The crude enzyme was stirred with ice-cold 0·1M-sodium pyrophosphate buffer, pH8·3 (10–12mg./ml.), and undissolved solid removed by centrifugation at 2°. Oxidation of D-amino acids was measured manometrically with O_2 as the gas phase. The main compartment of the Warburg vessel contained enzyme solution (1ml.), catalase suspension (5·0μl.), FAD (0·1ml. of a 10mM solution) and water (0·5ml.). Substrate and an appropriate D-amino acid (each in 0·2ml. of water) were placed in the first and second side arm respectively. The enzyme preparation did not catalyse the oxidation of glycine, D-glutamic acid or D-α-aminoadipic acid.

Measurements of radioactivity. Radioactivity was measured with a gas-flow counter (Nuclear–Chicago Corp., Des Plaines, Ill., U.S.A.). Samples were counted at infinite thinness as described by Trown *et al.* (1962).

Purification of [^{14}C]*penicillin N.* Mycelium of *Cephalosporium* sp. mutant 8650 was harvested 72hr. after inoculation from eight 500ml. shake flasks (each containing 100ml. of culture) and washed with water. The damp-dry mycelium was distributed in equal amounts into four 500ml. shake flasks each containing water (90ml.), DL-methionine (2mg./ml.) and DL-[1-^{14}C]valine (1·39μc/ml., 0·292μmole/ml.). The flasks were shaken at 135 rev./min. (2in. throw) and 27·5° for 8hr. The filtered suspension fluid and washing (500ml., pH7·4) contained 42μg. of penicillin N/ml. but less than 3·5μg. of cephalosporin C/ml. (biological assay). It was freeze-dried to give a powder (1·67g.) containing 12·6μg. of penicillin N/mg. The powder was extracted three times with 15ml. of 70% (v/v) ethanol and then once with 5ml., the solid being removed by centrifugation. Much of the ethanol was removed from the combined supernatants by distillation *in vacuo* on a rotary evaporator. Water (10ml.) was then added and the solution freeze-dried to give a powder containing 31μg./mg. of penicillin N. The total amount of penicillin N was 20·5mg. (yield 97%). Purification of this product was carried out by a chromatographic method similar to that used by Flynn, McCormick, Stamper, DeValeria & Godzeski (1962) for the isolation of isopenicillin N.

The product was dissolved in water (1·5ml.) and propan-2-ol (3·5ml.) was added. The mixture was shaken and the top layer removed. 70% (v/v) Propan-2-ol (3ml.) was added to the residue and the process repeated. After two more similar extractions most of the propan-2-ol was removed from the combined extracts on a rotary evaporator, water (0·5ml.) was added and the solution freeze-dried. The residue was dissolved in water (0·6ml.), propan-2-ol (1·4ml.) added and insoluble material removed by centrifugation. The supernatant was applied to a column (43cm.×1cm. diam.) of Sephadex G-25 (fine) packed in 70% (v/v) propan-2-ol and surrounded by a jacket of water at 8–12°. The column was developed with 70% (v/v) propan-2-ol, fractions of 2·5ml. being collected every hour. Biological assay indicated that penicillin N (15mg.; 72% yield) was present in fractions 63–81. Measurements of extinction at 260mμ indicated that cephalosporin C (approx. 1mg.) was present in fractions 68–88. Fractions 67–77 were pooled, much of the propan-2-ol was removed by rotary evaporation and the residual solution diluted with water and freeze-dried. The resulting preparation of [^{14}C]penicillin N was a white powder, about 70% pure by biological assay, with a specific radioactivity 0·39μc/mg. A radioautograph was made after electrophoresis of a sample on paper at pH4·5 followed by chromatography in butan-1-ol–acetic acid–water. Subsequent counting of the radioactive spots indicated that 80% of the radioactivity was associated with penicillin N, and 18% with a compound which behaved like penicillin N penicilloate. About 2% of the radioactivity was found in the area occupied by cephalosporin C (Fig. 6).

[1-^{14}C]Valine and methionine emerged from the Sephadex column in fractions 16–25. Methionine crystallized from fractions 18–22. It showed $[\alpha]_D^{20}+15°$ in 2N-HCl (c 1·1) and was therefore predominantly the L-isomer, although DL-methionine had been added to the suspension.

Mycelial nitrogen. The ninhydrin method was used to estimate amino N and ammonia N combined in acid hydrolysates of mycelium. Samples of mycelium dried at 110° (5–40mg.) were suspended in 6N-HCl (1–5ml.) in hard-glass tubes. The tubes were sealed, heated at 37° for 48hr. with occasional shaking to solubilize the mycelium partially and then held at 110° for 24hr. After removal of the HCl, the hydrolysates were shaken with water (1ml./mg. dry wt.). Any insoluble material was removed by centrifugation.

Ninhydrin colour yields (Moore & Stein, 1954) were determined on 0·05ml. samples of the hydrolysates with leucine as a standard. The N values obtained varied from 73 to 70mg. of N/g. dry mycelium with growing cultures. The value obtained for the inoculum (sporulating submerged culture) was 56mg. of N/g. dry wt. These values are similar to those found by Bent & Morton (1964) for *P. chrysogenum* by the Kjeldahl method (conidia, 56·7mg. of N/g. dry wt.; mycelium 80·4mg. of N/g. dry wt.).

RESULTS

Antibiotic production in a chemically defined growth medium. The production of penicillin N and cephalosporin C by *Cephalosporium* sp. C-91 and the change in mycelial weight with time are shown in Fig. 1. The change in mycelial nitrogen paralleled the change in mycelial weight. Qualitatively similar curves were obtained with mutant 8650. With both organisms the production of penicillin N and cephalosporin C began in the early stages of the growth of the culture but continued for 40hr. or more after net growth had ceased (Fig. 1). The rates of antibiotic production per unit weight of mycelium reached a maximum about 45hr. after inoculation.

The two mutants produced similar amounts of penicillin N, but C-91 produced considerably more cephalosporin C than did 8650. In neither case was there a clear-cut difference between the time-course of penicillin N production and that of cephalosporin C, at least during the first 72hr. However, in a series of fermentations with mutant 8650, in which the yield of penicillin N at 72hr. varied from 0·1 μmole/ml. to 1·0 μmole/ml., increases in the values for penicillin N tended to be associated with much smaller increases in the values for cephalosporin C. Fig. 2 shows the experimental values and calculated regression lines of N on C (*A*) and C on N (*B*).

At 24hr. after inoculation the new mycelial growth was mainly in the form of long hyphae with few septa apparent. By 48hr., segmentation of the mycelium was beginning and this was pronounced after 72hr. when about 60% of the mycelial threads were entirely segmented and only about 5% contained no septa. At 120hr. considerable fragmentation of the mycelial chains was observed. Staining with Sudan IV indicated that lipid was laid down at the corners and along the walls of the cells between 24 and 72hr. after inoculation.

When the fermentation was continued beyond the time at which maximum yields of antibiotics were obtained, the concentrations of both penicillin N and cephalosporin C in the culture fluid generally decreased. An attempt was made to ascertain whether the loss of antibiotics was due to their incorporation into the organisms in the later stages of the fermentation. A solution of L[1-^{14}C]valine (20 μc, 4·2 μmoles in 0·5ml. of water) was added to a culture in a 250ml. shake flask 72hr. after inoculation and the fermentation continued for 1hr. Other experiments (Warren, Newton & Abraham, 1967) indicated that virtually all the radioactivity in the culture fluid after this time would be present in penicillin N, cephalosporin C and deacetylcephalosporin C. This culture fluid, whose radioactivity was determined, was added immediately to the damp-dry mycelium obtained by filtration from a sister flask to which no [^{14}C]-valine had been added and the fermentation continued in the latter flask as before. Samples (31·6 μl.) of filtrates from this flask were counted at intervals. Samples from a further flask, which

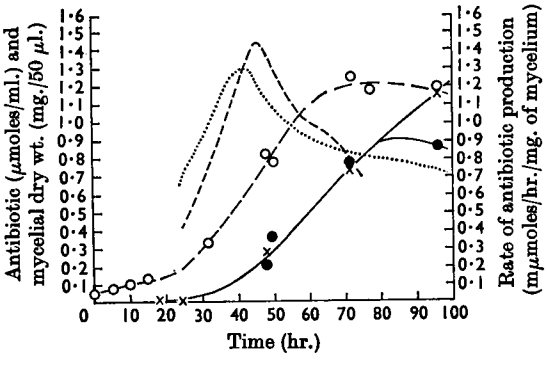

Fig. 1. Production of penicillin N and cephalosporin C in a chemically defined medium in shake flasks by *Cephalosporium* sp. C-91. --○--, Mycelial dry weight; —×—, penicillin N; —●—, cephalosporin C; ····, rate of increase in penicillin N per unit weight of mycelium; - - - -, rate of increase in cephalosporin C per unit weight of mycelium.

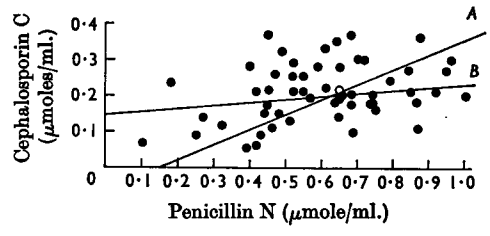

Fig. 2. Relationship between yields of penicillin N and cephalosporin C (●) produced 72hr. after inoculation by *Cephalosporium* sp. 8650 in a series of similar fermentations in shake flasks. Line *A* shows the regression of N on C (N=2·36C+0·16) and *B*, the regression of C on N (C= 0·084N+0·15), N and C being the antibiotic concentrations (μmole/ml.) and both lines being calculated by the method of least squares. The correlation coefficient (0·443) is significant at $P<0·001$. ○, Means of the values of N and C respectively.

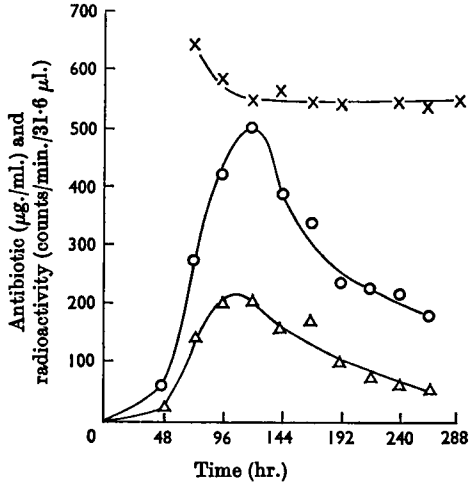

Fig. 3. Change with time of radioactivity from ^{14}C-labelled antibiotics and of antibiotic concentrations during fermentations in shake flasks with *Cephalosporium* sp. 8650. ×, Radioactivity; ○, penicillin N; △ cephalosporin C.

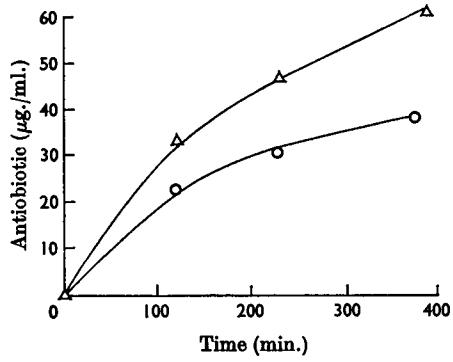

Fig. 4. Biosynthesis of antibiotics by suspensions (5ml.) of washed mycelium of *Cephalosporium* sp. 8650 in shake flasks (50ml.). △, Penicillin N; ○, cephalosporin C.

had remained undisturbed throughout the fermentation, were used for biological assay of penicillin N and cephalosporin C. The results of these measurements (Fig. 3) showed that the fall in the concentration of penicillin N and cephalosporin C was not accompanied by a similar fall in radioactivity. The decrease in the radioactivity of the culture fluid observed 24hr. after the initial measurement was made may be attributed to dilution of the ^{14}C-labelled culture fluid with unlabelled culture fluid trapped in the damp-dry mycelium to which the former was added.

Antibiotic production by washed mycelium. The formation of penicillin N and cephalosporin C in a suspension in water of washed mycelium of *Cephalosporium* sp. 8650 is shown in Fig. 4. Biosynthesis of penicillin N and cephalosporin C continued at a similar rate for at least 1–2hr. after the mycelium was harvested. It then normally continued at a slower, but substantial, rate for a further 8hr. and in some cases for a further 24hr. Similar results were obtained with mutant C-91. The production of antibiotics by suspensions of washed mycelium which had been stored at −20° for up to 4 days did not differ significantly from that by the same batch of mycelium which was resuspended immediately after harvesting.

Suspensions of washed mycelium which had been homogenized frequently produced up to 50% more cephalosporin C than did control suspensions within 5–10hr., and also showed a significant, though smaller, increase in the production of penicillin N. These increases may have been due to an increase in the efficiency of aeration after breakages in the long mycelial chains during homogenization. Although the appearance of the cells under phase contrast was not obviously changed, the mean length of the chains before homogenization was 30μ (range 11–105μ), whereas after homogenization it was 9·5μ (range 2·25± 22·5μ). When the efficiency of aeration was lowered by increasing the volume of suspension in similar shake flasks, the yield of penicillin N after 5hr. increased and that of cephalosporin C decreased (Table 1). When the speed of shaking was decreased from 200 to 60 rev./min. with a constant volume per flask, the penicillin N produced was reduced by 50% and cephalosporin C production ceased.

No clear-cut effects on the production of penicillin N and cephalosporin C by washed mycelium were produced when suspensions in water were replaced by a suspension in sodium phosphate–potassium phosphate buffer, in the buffered salt mixture described by Demain (1963*a*), or in a solution containing DL-α-aminoadipic acid, L-cysteine and valine (2mg./ml.). However, addition of DL-methionine (2mg./ml.) to suspensions commonly resulted in an increase of threefold or more in the quotient penicillin N produced/cephalosporin C produced. This change usually reflected an increase in the production of penicillin N, a decrease in that of cephalosporin C, and an increase in the total amount of the two antibiotics. Two experiments indicated that D-methionine was more effective than the L-isomer in these respects.

Antibiotic production by washed mycelium after ultrasonic disintegration. The production of antibiotics was studied with suspensions obtained by subjecting washed mycelium of *Cephalosporium* sp. 8650 to homogenization in water or the buffer used by Demain (1963*a*), followed by ultrasonic treat-

Table 1. *Production of penicillin N and cephalosporin C by different volumes of mycelial suspensions in shake flasks*

Suspensions of 1g. of damp-dry mycelium (mutant 8650) in distilled water (6ml.) were shaken in flasks (50ml.) at 27·5°. Yields for each antibiotic are expressed as percentages of the average yield for that antibiotic from 5 or 5·5ml. of suspension in the same experiment. In most cases the values represent the average of those from duplicate flasks. Variations between flasks were not greater than 10%.

Expt. no.	Volume of suspension (ml.)	Time of shaking (min.)	Yield (%)		Penicillin N / Cephalosporin C
			Penicillin N	Cephalosporin C	
1	5·0	300	100	100	0·87
	10·0	300	173	81	1·88
	15·0	300	224	74	2·5
2	5·5	360	100	100	1·43
	15·0	360	223*	29*	10·8*

* Values from one flask only.

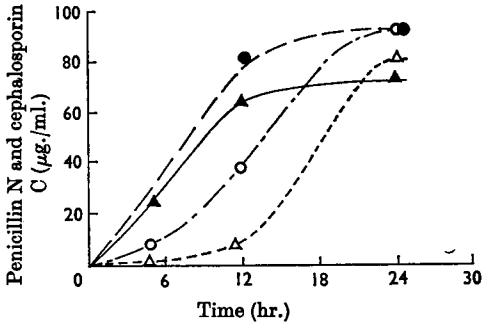

Fig. 5. Production of antibiotics by suspensions of *Cephalosporium* sp. C-91 after homogenization and ultrasonic treatment. (1) Homogenates: ●, penicillin N; ▲, cephalosporin C. (2) Homogenates treated ultrasonically for 30min. (see the Methods section): ○, penicillin N; △, cephalosporin C.

ment for varying periods of time. With suspensions obtained by ultrasonic treatment for 5, 10 and 30min., the yields of penicillin N after 10hr. were in most cases about 80, 70 and 20% respectively of those in the homogenate. The corresponding yields of cephalosporin C were about 66, 30 and less than 15% respectively. After ultrasonic treatment for 30min., however, there was a lag in antibiotic production for up to 10hr., which was succeeded by a phase of rapid antibiotic production, and by 24hr. the concentrations of penicillin N and cephalosporin C were as high as those in the homogenate. Similar results were obtained with mutant C-91 (Fig. 5). With both organisms the concentration of antibiotics subsequently declined, but more rapidly in the ultrasonically treated suspensions than in the homogenates. In a single experiment in which ultrasonic treatment was continued for 60min. no antibiotic production was detected.

Centrifugation for 5min. at 2500*g* of suspensions after ultrasonic treatment for 30min. separated them into a sediment and a cloudy supernatant. The sediment resuspended in the original buffer produced less than 20% of the amount of penicillin N produced by the whole sound-treated suspension and less than 10% of the amount of cephalosporin C. The supernatant alone synthesized no detectable amount of either antibiotic. The production of antibiotics, after a long lag, by whole suspensions that had been subjected to ultrasonic treatment was accompanied by morphological changes in the disrupted mycelium.

Morphological and other changes in disrupted mycelium. As observed under phase contrast, the homogenized mycelium consisted of threads (2·25–22·5μ) most of which were divided into separate cells by up to 10 septa. Ultrasonic treatment for increasing periods of time resulted in a rapid fragmentation of the threads. After 30min. most of the fragments that remained enclosed by cell walls contained only from one to two cells and these cells appeared to contain fewer granules than those of the original mycelium. In many cases a terminal cell was open at the far end and apparently empty, as though a previously contiguous cell had been removed with a septum. Other fragments consisted only of cylindrical cell walls sometimes with a septum. The morphological changes that occurred as the time of treatment with ultrasound was increased were reflected in changes in the total number of discrete mycelial fragments and in the number of colonies that grew when samples of the suspension were plated (Table 2). The total counts increased rapidly for 5min. as the mycelial chains were broken and then much more slowly. The colony counts appeared to reach a maximum after 15min. and then declined. After 30min. about 4·7% of the mycelial fragments appeared to be viable.

Table 2. *Change in the total number of discrete mycelial fragments and in the number of viable fragments after ultrasonic treatment*

The mycelium (mutant C-91) was subjected to ultrasonic treatment in buffer after homogenization (for details see the text). Samples were counted under dark-ground illumination in a Helber bacterial chamber after suitable dilution in water. The particles counted were those that contained apparently intact cells or disrupted cells containing recognizable fragments of cell wall or both. After dilution in water, viable counts were made as described in the Methods section. The length of individual cells (separated by septa) was approx. 2·25 μ.

Time of ultrasonic treatment (min.)	10^{-6} × No. of discrete mycelial fragments/ml.		10^{-6} × Change in number of discrete fragments/ml.		Mean no. of intact and/or damaged cells per fragment
	Total	Viable	Total	Viable	
0	95	21	—	—	4·2 (range 1–10)
1	165	39	+ 70	+18	
2	220	51	+125	+30	
5	292	53	+197	+32	
15	315	55	+220	+34	
30	366	17	+271	− 4	1·2 (range 1–2)

Table 3. *Uptake of material liberated from mycelium by homogenization and ultrasonic treatment*

Suspensions of mycelium (mutant C-91) in water were subjected to homogenization and to homogenization followed by ultrasonic treatment for 30 min. as described in the Methods section. Samples (6 ml.) of homogenates and ultrasonically treated material were shaken in flasks (50 ml.) at 200 rev./min. and 27°. Flasks were harvested at different times and their contents centrifuged at 2500*g* for 15 min. Samples of the superantants were freeze-dried and the residues weighed. All supernatants from homogenized material were nearly clear. Supernatants from ultrasonically treated material were initially slightly opalescent, but were clear when taken from flasks which had been shaken for more than 12 hr. Sediments obtained after centrifugation were washed with a little water on to a filter, sucked damp-dry and weighed. They were then dried at 105° and reweighed.

Time of resuspension (hr.)	Non-volatile material in supernatants (mg./ml.)		Dry wt. of sediment (mg./ml. of culture)	
	After homogenization	After homogenization and ultrasonic treatment	After homogenization	After homogenization and ultrasonic treatment
0	5·0*	12·0	15·3	7·8
3	2·8	11·5	15·6	8·7
10	2·5	7·0	15·8	11·8
24	3·0	2·7	14·8	10·0

* Before homogenization the corresponding weight was < 0·2 mg.

Colony counts carried out after resuspension for 24 hr. in shake flasks showed an approx. twofold increase in the number of viable particles in homogenized mycelium. No corresponding increase was found under the same conditions, in the number of viable particles in mycelium subjected to ultrasonic treatment.

Relatively little change in morphology was observed when suspensions which had been treated ultrasonically for 30 min. were incubated in shake flasks for periods varying from 5 to 10 hr.; but within this time non-septate hyphal tubes were seen to be protruding from occasional cells. After 24 hr., however, when antibiotic production had reached its maximum, the number of growing hyphae had greatly increased. This growth was accompanied by a disappearance from the supernatant fluid of much of the material liberated from the mycelium by ultrasonic treatment and a gain in the dry wt. of the sedimentable material (Table 3). The increase in the dry wt. of the latter was accompanied by a proportionally greater increase in its water content. Thus after 10 hr. the quotient damp-dry wt./dry wt. had risen from 7·8 to 12·2.

Table 4. *Extracellular and intracellular concentrations of penicillin N and cephalosporin C*

Mycelium (mutant 8650) was harvested after growth for different times in a chemically defined medium in shake flasks (500ml.). Antibiotics were estimated by antibacterial assay. Intracellular antibiotics were extracted with cold 70% (v/v) ethanol and water (see the Methods section). Intracellular concentrations are those calculated on the assumption that the antibiotics were freely diffusable through an intracellular volume of 0·63ml./g. of damp-dry mycelium. The amounts of intracellular antibiotics/g. of damp-dry mycelium are thus 0·63 times the values given for intracellular concentrations. Although the antibiotics have been assayed as penicillin N and cephalosporin C respectively, further studies are needed to distinguish them from the corresponding iso compounds with side-chains derived from L-α-aminoadipic acid instead of from the D-isomer.

Time of growth (hr.)	Concn. of penicillin N (μmoles/ml.)			Concn. of cephalosporin C (μmoles/ml.)		
	Extracellular (*A*)	Intracellular (*B*)	$\frac{A}{B}$	Extracellular (*A*)	Intracellular (*B*)	$\frac{A}{B}$
48	0·28	0·036	7·7	0·095	0·013	7·2
72	0·89	0·047	19	0·21	0·016	13·4
96	1·26	0·062	20	0·46	0·04	11·4

The weights of material liberated from the mycelium by homogenization and ultrasonic treatment respectively were paralleled by the extinction at 260mμ of clear supernatants obtained by centrifugation at 16000*g* for 20min., $E^{1\%}_{1\,cm.}$ for the material in the supernatants being approx. 30. Paper chromatography and electrophoresis revealed that the material also contained a mixture of amino acids and other ninhydrin-positive substances. During resuspension in shake flasks there was a decrease in the extinction at 260mμ of the supernatant fluid and also in the ninhydrin-positive material present.

Mycelium which had been homogenized and subjected to ultrasonic treatment was ground with glass beads in the manner described by Demain (1963*a*). After grinding, the number of viable fragments fell to about 1% of the number present after ultrasonic treatment. However, such ground mycelium failed to produce detectable amounts of penicillin N or cephalosporin C when suspended in buffer in shake flasks for 24hr.

Intracellular and cell-bound compounds. Apparent concentrations of certain intracellular or cell-bound compounds or both were calculated on the assumption that these compounds were distributed uniformly through a volume equal to that of the intracellular water and that the latter was 0·63ml./g. of damp-dry mycelium. This value was derived from the loss of weight of the damp-dry mycelium (79%) on drying at 105° and an arbitrary correction for loss of intercellular water. It is similar to that estimated for the intracellular volume of *E. coli* (Roberts, Abelson, Cowie, Bolton & Britten, 1955).

Penicillin N, cephalosporin C, 6-aminopenicillanic acid and 7-aminocephalosporanic acid. Apparent intracellular and extracellular concentrations of penicillin N and cephalosporin C, as estimated by antibacterial assay with mycelium harvested at different times during fermentation in the chemically defined medium, are shown in Table 4. There appeared to be some increase in the intracellular concentration of both substances between 48hr. and 72hr. or 96hr. after inoculation, but this was much smaller than the rise in extracellular concentrations. The quotients extracellular concentration/intracellular concentration varied between 7 and 20.

In an experiment to ascertain whether a detectable amount of penicillin N could enter the mycelial cell from the extracellular fluid, [^{14}C]penicillin N (0·4mg., 0·1365μc in 0·1ml. of water) was added to 2ml. of a suspension of washed mycelium (1g. of damp-dry mycelium/6ml.) in a Warburg vessel. The flask was shaken in oxygen for 8hr. Less than 0·4% of the added radioactivity was present in a 70% (v/v) ethanol extract of the filtered washed mycelium. Analysis of the extracellular fluid by radioautography after paper chromatography and electrophoresis indicated that less than 0·5% of this radioactivity could be attributed to a compound which behaved like cephalosporin C. Virtually all the added [^{14}C]penicillin N was recovered. The positions of penicillin N, cephalosporin C and other compounds after electrophoresis and chromatography on paper are shown in Fig. 6.

Material from culture fluids containing penicillin N and cephalosporin C and also from the intracellular pool was subjected to paper electrophoresis and chromatography and phenylacetylation was then carried out on the paper (Loder, Newton & Abraham, 1961). Subsequent bioautography gave no indication of the presence of 6-aminopenicillanic acid or of 7-aminocephalosporanic acid. The method used would have detected 0·008μmole of the former compound and 0·03μmole of the latter in 1g. of damp-dry mycelium.

Amino acids. The amounts of some of the amino

Table 5. *Intracellular amino acids in mycelium harvested at different times after inoculation*

Mycelium from *Cephalosporium* sp. mutant 8650 was harvested from cultures in shake flasks (250ml.). Some of the amino acids extracted by hot 70% ethanol were estimated with an amino acid analyser as described in the Methods section. In a similar experiment mycelial damp-dry weights after 18, 46, 73 and 105hr. were 0·56, 3·05, 3·75 and 3·7g. respectively from 45ml. of culture and the weights of crude amino acid mixtures obtained were 3·2, 32, 38 and 75mg. respectively.

Amino acid / Time of harvesting (hr.).	Amount (μmoles/g. of damp-dry cells) in mycelium			
	18	46	73	120
Proline	2·99	1·22	3·41	7·72
Glutamic acid	1·24	3·55	4·04	5·19
Aspartic acid	0·61	0·44	0·59	0·44
α-Aminoadipic acid	0·24	0·17	0·19	0·43
Lysine	0·35	0·13	0·15	0·47
Valine	0·16	0·17	0·37	0·56
Methionine	1·39	1·52	1·86	4·77
Alanine	1·58	2·07	2·68	2·32
Serine	1·19	1·37	2·06	1·70

Table 6. *Amino acid composition of different fractions of mycelium*

Mycelium (mutant 8650) was harvested after growth in defined medium for 72hr. in shake flasks and fractionated as described in the Methods section. The amounts of individual amino acids in each fraction are given as moles/100moles of the total amino acids estimated in the fraction concerned. — Signifies not detected.

Amino acid	Composition			
	Intracellular pool	Hot-trichloro-acetic acid extract	'Protein' hydrolysate	Hydrolysate of residual mycelium
Proline	31·3	4·2	6·3	6·0
Glutamic acid	26·1	7·7	11·9	7·6
Aspartic acid	2·2	11·3	12·3	8·7
α-Aminoadipic acid	0·6	—	—	—
Lysine	1·8	38·3	6·1	3·9
Glycine	2·8	8·2	10·9	11·1
Alanine	14·2	6·5	10·6	10·7
Valine	2·2	3·3	6·2	7·9
Leucine	1·4	3·1	8·9	13·1
Isoleucine	1·2	1·9	4·4	6·7
Methionine	*	*	1·2	1·1
Cyst(e)ine	†	†	†	†
Serine	11·8	6·6	6·0	5·8
Threonine	0·9	1·5	5·4	4·9
Arginine	0·8	2·7	3·7	4·1
Histidine	0·3	0·9	2·2	1·3
Phenylalanine	0·7	2·4	4·0	6·1
Tyrosine	—	—	—	—

* Present but not resolved from an uncharacterized compound.

† Present but not resolved from penicillaminic acid in the performic acid-oxidized sample.

acids in the intracellular pool of mycelium harvested at different times from fermentations in shake flasks in a chemically defined medium are shown in Table 5. The size of the pool increased between 48hr., when the phase of rapid growth was approaching its end, and 120hr. The amino acids present in relatively high concentrations were glutamic acid, proline, methionine, alanine and serine. Valine and α-aminoadipic acid were present in much lower concentrations. However, these concentrations were more than one order higher than those of intracellular penicillin N and cephalosporin C.

Less than 2% of the material extracted with hot

trichloroacetic acid from the mycelial residue after extraction with 70% (v/v) ethanol consisted of amino acids. The major amino acid in this hot-trichloroacetic acid extract was lysine and that present in the next highest amount was aspartic acid. Valine was present in much smaller amounts (Table 6). No α-aminoadipic acid was detected in the hot-trichloroacetic acid extract, or in acid hydrolysates of the mycelial protein fraction or of the mycelial residue.

A sample (21·4mg.) of the crude amino acid pool was used as substrate for D-amino acid oxidase. No oxygen uptake was observed although less than 1μmole of amino acids susceptible to oxidation (Burton, 1955; Wellner & Meister, 1961) was detectable under the conditions used. When a second sample (10·7mg.) reacted with L-amino acid oxidase, oxygen uptake corresponded to 16·5μ-moles of L-amino acid.

Several ninhydrin-positive substances in the amino acid pool were not characterized or were not completely resolved from each other by the amino acid analyser under the conditions used. The nature of some of these substances was revealed by paper electrophoresis and chromatography of samples (usually about 500μg.) of 70% (v/v) ethanol extracts. Electrophoresis at pH4·5 revealed a number of substances which migrated towards the anode. The major ninhydrin-positive spot was not resolved from glutamic acid, penicillin N and cephalosporin C. Smaller spots were present in the positions of α-aminoadipic acid, aspartic acid and glutathione disulphide respectively (Fig. 6). Minor compounds migrated slightly faster than α-aminoadipic acid and slightly slower than glutamic acid respectively. These compounds were shown to behave like saccharopine (Kjaer & Larsen, 1961) and glutathione respectively. A minor compound which migrated slightly less far than glutathione disulphide was shown to be saccharopine lactam (Fig. 6). Three minor compounds which migrated towards the cathode at pH4·5 were identified as β-alanine, γ-aminobutyric acid and δ-aminovaleric acid respectively. γ-Aminobutyric acid migrated slightly less far than δ-aminovaleric acid and about 0·5 times as far as lysine.

After oxidation with performic acid of the crude mixture of substances extracted from the intracellular pool electrophoresis was carried out at pH1·8. Under these conditions at least two ninhydrin-positive substances were found to migrate towards the anode. The substance responsible for the major spot was glutathione sulphonic acid. A second spot, about one-fifth of the size of that due to the latter, was due to cysteic acid, which migrated about 2·5 times as far as glutathione sulphonic acid. In some experiments two smaller spots were detected slightly nearer to the origin than those due to cysteic acid and glutathione sulphonic acid respectively. That close to cysteic acid corresponded in position to penicillaminic acid, and that close to glutathione sulphonic acid to the sulphonic acid of δ(α-aminoadipoyl)cysteinylvaline which had been prepared from an authentic sample of the all L-disulphide kindly provided by Professor Rudinger. The material responsible for this latter spot is referred to as 'ACVSO$_3$H'. However, preliminary studies have shown that it yields glutamic acid and glycine as well as α-aminoadipic acid, cysteic acid and valine on hydrolysis and have indicated that it consists of a mixture of related peptides. A major spot near the origin was due to a substance which behaved like taurine. The remainder of the ninhydrin-positive substances, which presumably did not contain a sulphonic acid grouping, migrated towards the cathode.

A semi-quantitative estimate was made of the relative amounts of some of the ninhydrin-positive substances in the intracellular pool of mycelium harvested 72hr. after inoculation and resuspended in water in shake flasks for up to 4hr. During this period no clear change was apparent in the pool from visual observations of spots coloured with ninhydrin.

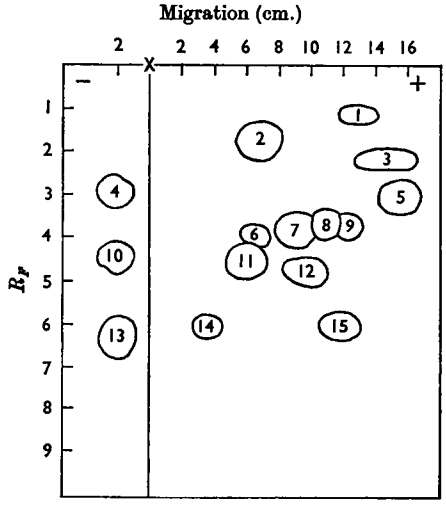

Fig. 6. Composite map of the positions of compounds on Whatman no. 4 paper after they had been subjected to (*a*) electrophoresis in pyridine acetate buffer (pH4·5) at 4kv for 35min.; (*b*) paper chromatography, with butan-1-ol–acetic acid–water (4:1:5, by vol.) for 6hr. at 4°. (1) GSSG, (2) saccharopine, (3) saccharopine lactam, (4) Gly, (5) Asp, (6) 7-aminocephalosporanic acid, (7) Glu, (8) deacetylcephalosporin C, (9) penicilloate of penicillin N, (10) Pro, (11) α-aminoadipic acid, (12) cephalosporin C, (13) Val, (14) 6-aminopenicillanic acid, (15) penicillin N.

DISCUSSION

In the chemically defined growth medium used here the rate of production of penicillin N and cephalosporin C per unit weight of mycelium passed its maximum as the thin non-septate hyphae of early growth were becoming thicker and septate. However, throughout the fermentations the mycelium of the *Cephalosporium* sp. showed varying degrees of heterogeneity and further studies are required to ascertain the relationship between the morphologically distinguishable states of the mycelium and the ability of the latter to synthesize the antibiotics at a maximum rate.

Whether enzyme-catalysed reactions were involved in the decline in the concentrations of the two antibiotics after they had reached maximum levels in the culture fluid was not established. A similar phenomenon with bacitracin has been attributed to the incorporation of the latter into the spore coat of *Bacillus licheniformis* (Bernlohr & Novelli, 1963) although this interpretation has not been confirmed (Snoke, 1964). Evidence was obtained that the fall in concentration of the cephalosporins was not accompanied by an uptake of these substances by the *Cephalosporium* sp. and α-aminoadipic acid was not detected in any mycelial fraction except the intracellular pool.

Analysis of the variations in the amounts of penicillin N and cephalosporin C produced in a series of similar shake-flask fermentations by *Cephalosporium* sp. 8650 showed a positive correlation between the yields of the two antibiotics. The biosynthesis of the cephalosporin C molecule requires oxidative processes additional to the oxidative condensations leading to penicillin N. The correlation suggested that a certain balance between enzyme systems involved in the first and second antibiotic respectively tended to be maintained in *Cephalosporium* sp. 8650 during considerable variations in the activities of the enzymes in the mycelium.

An indication of a linkage between the biosynthetic pathways leading to penicillin N and cephalosporin C respectively came from experiments with suspensions of washed mycelium in shake flasks. Moderate decreases in aeration, brought about by increases in the volume of culture fluid per flask, resulted in a fall in the yield of cephalosporin C, and a rise in the yield of penicillin N. They also resulted in a rise in the yield of both antibiotics together, which should not have occurred if the change in the relative amounts had only reflected the redeployment of a common intermediate in limited supply. This raises the possibility that an enzyme concerned specifically with penicillin N formation can be partially repressed under conditions which facilitate the formation of cephalosporin C.

The addition of methionine to suspensions of washed mycelium caused changes in the yields of penicillin N and cephalosporin C which were qualitatively similar to those produced by moderate decreases in the efficiency of aeration. The present experiments throw no light on the mechanism by which these changes occur, but D-methionine, which was more effective than the L-isomer in producing them, was also metabolized more extensively by the mycelium.

The ability of suspensions of washed mycelium of the *Cephalosporium* sp. to synthesize antibiotics without the addition of any energy or nitrogen source to the extracellular fluid appeared to be paralleled by an ability to maintain an amino acid pool at a nearly constant level. No significant proportion of the pool consisted of D-amino acids susceptible to oxidation by D-amino acid oxidase, in contrast with the amino acid pool in *Bacillus brevis*, at least one-seventh of which was reported to consist of D-isomers (Okuda, Uemura, Bodley & Winnick, 1964). The pool from the *Cephalosporium* sp. was characterized by relatively high concentrations of glutamic acid, proline and alanine, which are readily derived from members of the tricarboxylic acid cycle, but relatively low concentrations of three amino acids, α-aminoadipic acid, cyst(e)ine and valine, which are precursors of penicillin N and cephalosporin C. However, although the concentration of cyst(e)ine was low, those of serine and taurine (after oxidation with performic acid) were relatively high. The interrelationship of these amino acids may be connected with the ability of methionine to stimulate the production of penicillin N and cephalosporin C by the *Cephalosporium* sp. in a growth medium. Demain *et al.* (1963) suggested that methionine raises the concentration of cysteine by repressing the synthesis of cystathionase, an enzyme able to degrade cysteine to pyruvate (Rowbury & Woods, 1964).

The presence of saccharopine, as well as lysine, in the amino acid pool, is consistent with the assumption that the former is an intermediate in the formation of L-lysine from L-α-aminoadipic acid in this organism, as it appears to be in *Neurospora crassa* (Jones & Broquist, 1965). And the presence of a substance that appears to be δ-aminovaleric acid indicates that L-α-aminoadipic acid can undergo decarboxylation as well as conversion into L-lysine.

Relatively very small amounts of penicillin N and cephalosporin C (or of substances that behaved like them on biological assay) were extracted from the mycelium. Hence the antibiotics were either excreted against a concentration gradient or they were confined to a small fraction of the total cell

volume, and an experiment with [^{14}C]penicillin N suggested that virtually none of the extracellular penicillin N re-entered the mycelium. Other work with *P. chrysogenum* has indicated that the extracellular benzylpenicillin produced during fermentation reaches much higher concentrations than the intracellular concentration of this antibiotic (Demain, 1957).

A possible intermediate in the biosynthesis of penicillin N and cephalosporin C is the tripeptide δ-(α-aminoadipoyl)cysteinylvaline, which has been obtained in the form of its sulphonic acid from the mycelium of *P. chrysogenum* by Arnstein & Morris (1960). Material that behaved like this sulphonic acid on electrophoresis at pH 1·8 was only just detectable by the ninhydrin reaction when extracts of the *Cephalosporium* sp. were treated with performic acid and preliminary experiments indicated that this material was not homogeneous. Thus the tripeptide did not accumulate in the cell in an amount comparable with that of glutathione. 6-Aminopenicillanic acid and 7-aminocephalosporanic acid were not detected in mycelial extracts by the procedures used. If any of these compounds function in the free state as intermediates in the biosynthesis of penicillin N or cephalosporin C they reach only very low concentrations in the cells, like the antibiotics themselves.

Demain (1963*a*) suggested that the use of mycelium broken by ultrasonic treatment might represent the first step towards a cell-free system able to synthesize cephalosporin C. The results of experiments described here with mycelium subjected to ultrasonic treatment were qualitatively similar to those reported previously (Demain, 1963*a*). However, the lag before rapid antibiotic production began was nearly 12hr. Most of the amino acid pool of the mycelium appeared in the supernatant fluid on ultrasonic treatment and the mycelial chains underwent extensive fragmentation with disruption of many individual cells. After the lag the amino acids disappeared in large measure from the supernatant and it appeared that permeability barriers had been re-established. At the same time new hyphae were seen to be growing from cells of swollen or distorted appearance. Thus the system used in these experiments in which penicillin N and cephalosporin C were synthesized was not cell-free. The conditions under which cells damaged by ultrasonic treatment can re-form the systems necessary for antibiotic production required further study.

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