Incorporation of Acetate into Cephalosporin C

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Cephalosporin C, an antibiotic with structure (I) (Abraham & Newton, 1961; Hodgkin & Maslen, 1961), was first isolated from an impure preparation of the chemically related penicillin N‡ [(D-4-amino-4-carboxybutyl)penicillin] that had been obtained from the culture fluid of a *Cephalosporium* sp. (Newton & Abraham, 1956; Abraham & Newton, 1956). The amount of cephalosporin C produced by this organism, which had been isolated by Brotzu (1948), was too low for the antibiotic to be detected by antibacterial assay in the culture fluid. Much higher yields of the antibiotic were subsequently obtained from a mutant of the original *Cephalosporium* sp. (Miller, Kelly & Codner, 1961), and the use of the mutant strain enabled workable methods to be devised for the isolation of cephalosporin C from fermentation liquors.

With the original strain of *Cephalosporium* sp. the production of penicillin N in a medium containing sucrose and corn-steep liquor was increased by the addition to the medium of ammonium acetate (Florey *et al.* 1956). A similar culture medium containing ammonium acetate was shown to be useful for the production of cephalosporin C by the mutant strain by Miller *et al.* (1961). These findings raised the questions whether acetate was incorporated from the medium into penicillin N and whether it played a part in the biosynthesis of cephalosporin C other than that of providing the acetoxyl group in the molecule. When benzylpeni-

cillin was produced in a synthetic medium containing [1-¹⁴C]acetate, ¹⁴C was incorporated into the carboxyl group of the valine fragment of the molecule and also into the C=O group of the β-lactam ring (Tome, Zook, Wagner & Stone, 1953; Stevens & DeLong, 1958; Demain, 1959).

This paper describes the isolation of cephalosporin C produced by laboratory-scale fermentations in complex media containing [1-¹⁴C]acetate and records the results of degradations designed to provide information about the distribution of ¹⁴C in the labelled compound.

EXPERIMENTAL

Paper chromatography and electrophoresis

Paper chromatograms were run on Whatman no. 1 paper in butan-1-ol-acetic acid-water (4:1:4, by vol.). Except where otherwise stated, electrophoresis on Whatman no. 1 paper was carried out for 2.5 hr. at 14 v/cm. in collidine acetate (0.05 M with respect to acetate), pH 7.0, or for 30 min. at 83 v/cm. in pyridine acetate (0.05 M with respect to acetate), pH 4.5. With the latter the apparatus used was similar to that described by Katz, Dreyer & Anfinsen (1959).

Amino acids were detected on paper by coloration with ninhydrin. 6-Oxopiperidine-2-carboxylic acid and a number of other compounds were detected by the method of Rydon & Smith (1952). The Cl₂ was produced *in situ* in a glass tank (25 cm. × 20 cm. × 40 cm.) from 0.5 M-KMnO₄ (30 ml.) and 6 N-HCl (30 ml.), the procedure being similar to that of Reindel & Hoppe (1954) except that the concentration of Cl₂ was 1.9 m-moles/l. instead of 0.3 m-mole/l. The papers (dried for 1 hr. at 105°, or 3 hr. at 60°) were exposed to Cl₂ in the tank for 30 min. and subsequently hung in a strong draught for at least 1 hr. to remove excess of Cl₂. They were then sprayed with a 1% (w/v) starch solution, followed after 1 min. (as suggested by Dr G. T. Young) by a 1% (w/v) solution in KI.

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‡ We have agreed to adopt the name 'penicillin N' for the antibiotic previously known as 'cephalosporin N' or 'synnematin B'.

(I)

Fermentation methods

Fermentations in stirred aerated media. The first two fermentations to which sodium [^{14}C]acetate was added were carried out at the Antibiotics Research Station, Clevedon, Somerset. The first was with 16 l. of medium in 20 l. Pyrex-glass vessels. The aeration rate was 15 l./min. with a stirring speed of 1400 rev./min., a small paddle over perforated sparge tubes being used. Foaming was controlled initially by addition to the medium of a mixture (0.25%, v/v) of silicone A anti-foam (Midland Silicones Ltd., London) and tripentyl citrate (1:1, v/v). During the fermentation a mixture of silicone A and Risella oil (3:97, v/v) was added when necessary. The second fermentation was carried out in a smaller glass vessel with 3 l. of medium, a stirring speed of 900 rev./min. and an aeration rate of 9 l./min. The temperature was 26° in each case. With both fermentations the outflowing air was passed through a column packed with Berl saddles which contained 5N-KOH. The CO_2 absorbed in the KOH was precipitated as BaCO_3 .

Fermentations in shaken flasks. Subsequent fermentations were carried out at Oxford in 500 ml. conical flasks, each with four 1 cm. $\times$ 10 cm. straight indentation baffles on the sloping walls of the flask. The flasks, containing 100 ml. of medium, were plugged with non-absorbent cotton wool covered with cotton gauze, and then sterilized in an autoclave at 15 lb./in.² for 20 min. After suitable aseptic inoculation, they were mounted on a rotary shaker (1.5 in. throw at 160 rev./min.) enclosed in a box whose air temperature was thermostatically controlled. During the fermentation a slow stream of air was drawn through the box (which was fitted with an inlet and outlet tube) and then through two Dreschel bottles, each of which contained 150 ml. of 4N-NaOH. At the end of the fermentation the CO_2 absorbed in the NaOH was precipitated as BaCO_3 . Foaming was effectively controlled by coating the inside of the flasks with a thin film of silicone A anti-foam, which was applied as a 5% (w/v) solution in chloroform before sterilization. Unless otherwise stated, the detailed procedures described in the following sections are those used for fermentations in shaken flasks.

Maintenance of cultures and preparation of spore suspension. The organism used was a *Cephalosporium* sp., C.M.I. 49, 137, mutant 8650. Spores of the organism, suspended in the medium of Naylor & Smith (1946), were freeze-dried in ampoules and the latter sealed and stored at 4°. From these master cultures a spore inoculum was prepared by suspending the contents of an ampoule in a small amount of sterile distilled water, and were inoculated with a loop, from this suspension, slopes of half-strength Czapek-Dox agar (Dox, 1910) in 30 ml. screw-cap bottles. After incubation for 14 days at 24–26° the growth from these slopes was used to inoculate similar slopes of Le Page & Campbell (1946) medium (one-tenth strength) solidified with 2% (w/v) of agar. After a further incubation for 14 days at 24–26° these latter slopes could be stored for up to a month at 4° before use. The growth from these slopes was washed off with 5 ml. of sterile distilled water (the operation being assisted by the use of a few glass balls of about 5 mm. diam.) and the resulting spore suspension was used for the preparation of the inoculum.

Preparation of inoculum. The spore suspension (2 ml.), prepared as described, was added to 100 ml. of a medium

(medium A) designed to produce a dense mycelial growth containing many submerged spores. After growth for 70–72 hr. in shaken flasks at 27° the resulting culture (referred to below as 'the inoculum') was used to inoculate the medium for the final fermentation. The inoculum could be kept at 4° for up to 2 weeks without noticeable deterioration.

Medium A had the following composition: corn-steep liquor (Brown and Polson Ltd., Paisley, Scotland) to give 800 mg. of N/l. (27.2 g.); ammonium acetate, 4.4 g.; sucrose, 20.0 g.; tap water, 1 l. The medium was adjusted to give pH 6.5–6.8 after steam-sterilization at 15 lb./in.² for 40 min.

Production of cephalosporin C. Two media were used for the production of cephalosporin C (Miller *et al.* 1961). Medium B1 had the following composition: fish meal (Hull Fish Meal and Oil Co., Hull) to give 700 mg. of N/l. (7.8 g.); corn-steep liquor to give 50 mg. of N/l. (1.7 g.); meat meal (British Glues and Chemicals, Newcastle upon Tyne) to give 300 mg. of N/l. (3.4 g.); ammonium acetate, 1.9 g.; sucrose, 12 g.; glucose, 3 g.; DL-methionine, 0.5 g.; tap water, 1 l. The medium was adjusted to give pH 6.5–6.8 after steam-sterilization at 15 lb./in.² for 40 min. Medium B2 contained no meat meal, corn-steep liquor to give 41 mg. of N/l. and ammonium acetate at a concentration half that in B1. It contained all the other constituents of B1 in double concentrations and, in addition, 'distillers solubles' (The Distillers Co., Edinburgh) to give 300 mg. of N/l. (8.6 g./l.).

Medium B1 or B2 (100 ml.) was inoculated with 2 ml. of the inoculum. The maximum yield of cephalosporin C was obtained in about 70 hr.

Determination of acetate and anions of other weak acids in culture fluids. (a) For estimation of the amounts of volatile weak acid remaining in the culture fluid at various stages of the fermentation in shaken flasks, samples (1.7 ml.) were taken from each of 10 flasks and combined. After removal of the mycelium, the culture fluid (10 ml.) was passed through a column (1 cm. diam. $\times$ 6.5 cm.) of Dowex 50 (H^+ form; X4; 200–400 mesh) and the column washed with water (10 ml.). The combined effluents (pH 2.2), which showed no ninhydrin reaction, were freeze-dried. Titration of the distillate to pH 8 with 0.1N-NaOH gave a value for the total volatile acid, which included some strong acid (HCl). The titrated solution was freeze-dried, the residue dissolved in 2.2 ml. of water and a 2.0 ml. sample of the resulting solution titrated electrometrically to pH 2.5 with 0.05N-HCl added from a micrometer syringe. Analysis of the titration curve enabled the total amount of acetic acid and other weak acids to be estimated to within $\pm 5\%$ when this corresponded to a concentration of not less than about 1 m-equiv./l. in the culture fluid. To test for the presence of weak volatile acids other than acetic acid, samples of the neutralized distillates were analysed by: (1) chromatography in di-isopropyl ether on Whatman no. 1 paper treated with dimethylsulphoxide as described by Hammarberg & Wickberg (1960); (2) electrophoresis on Whatman no. 1 paper (about 100 v/cm. for 20 min.) in $(\text{NH}_4)_2\text{CO}_3$ buffer (7.9 g./l.), pH 8.9, as described by Gross (1958, 1961). Under the conditions used a volatile weak acid should have been detected if its concentration in the culture fluid were not less than 0.2 m-equiv./l.

(b) With the fermentations in stirred and aerated medium, 20 ml. samples of the culture fluid were mixed with 5 ml. of

syrupy H_3PO_4 and the volatile acid was removed by distillation in steam, through a splash trap, until 200 ml. of distillate had been collected. The acid in the distillate was titrated with standard alkali.

Bioassay of cephalosporin C. In addition to cephalosporin C, some penicillin N (Newton & Abraham, 1954) and traces of cephalosporin P (Burton & Abraham, 1951) were produced in the fermentations. Cephalosporin P was without action on the organism used for assay [*Salmonella typhi*, strain Mrs S. (Felix & Pitt, 1935)] at the concentrations at which it was present in culture fluids. To inactivate penicillin N (largely by conversion into its penillic acid) a sample of the culture fluid was brought to pH 2.5–3.0 with $\text{m-H}_3\text{PO}_4$ and kept at 37° for 3 hr. The pH of the stirred solution was then adjusted carefully to 6.5–7.0 with N-NaOH . The cephalosporin C in the sample was virtually unaffected by this process (Newton & Abraham, 1956) and was subsequently assayed by the hole-plate method (Brownlee *et al.* 1948). Pure cephalosporin C (sodium salt) (10 units/mg.) was used as a standard.

Isolation of cephalosporin C

In general, the contents of two shaken flasks were combined for the isolation of cephalosporin C. The culture (200 ml.) was adjusted to pH 5.5 with acetic acid and the mycelium and other suspended solids were removed by centrifuging. The cloudy supernatant liquid was decanted, and the solids were washed with 200 ml. of water. The combined supernatant and washings were brought to pH 2.8 by addition of Dowex 50 (H^+ form; X8). After removal of the resin by centrifuging, the supernatant liquid was kept at 37° for 3 hr. to convert penicillin N into its penillic acid.

The liquid was then concentrated *in vacuo* in a rotary evaporator (bath temp. 24°) to about 10 ml., when a white gum precipitated and was separated by centrifuging. The resulting clear dark-brown liquid, followed by 30 ml. of water, was passed through a column (1 cm. diam. $\times$ 3.1 cm.) of Amberlite IRA-400 (acetate form; 100–200 mesh). The size of the column was such that Cl^- and other inorganic anions were adsorbed and all the cephalosporin C appeared in the effluent. The effluent was freeze-dried to yield a brown powder. The latter was dissolved in 2 ml. of ammonium acetate buffer (0.2M), pH 5.0, prepared as described by Hirs, Moore & Stein (1952), and applied to a column (1 cm. diam. $\times$ 50 cm.) of Amberlite XE-58 resin (a finely divided form of Amberlite IR-4B resin, approx. 100–150 mesh) in the acetate form and in equilibrium with the ammonium acetate buffer. Elution was carried out with the same buffer supplied at a constant rate by a micropump (type 4501, LKB-Produkter, Stockholm), 2 ml. fractions being collected every 5 min. Samples taken from odd-numbered fractions were diluted appropriately with the buffer and the extinctions of the resulting solutions were measured at 260 μm (the ultraviolet-absorption spectrum of cephalosporin C shows E_{max} at 260 μm). When [$1\text{-}^{14}\text{C}$]acetate had been added to the fermentation the radioactivities of the same fractions were also measured.

On the basis of the curve obtained by plotting extinction against fraction number, suitable fractions were combined (in the example given in Fig. 2, fractions 95–170) and the solution was freeze-dried to remove most of the ammonium acetate. The contents of the flask were then dissolved in 2 ml. of water and applied to a column (1 cm. $\times$ 5 cm.) of

Dowex 50 resin (H^+ form; X8; 200–400 mesh). When the solution had passed into the column, the latter was quickly washed with water until the total volume of the washings was 15 ml. The resin was extruded, broken up in water (10 ml.) and the mixture stirred and titrated with N-NaOH to pH 6.5. The resin was filtered off, washed with water, and the combined filtrate and washings were freeze-dried, to yield a preparation of cephalosporin C (sodium salt) which readily crystallized when mixed with a minimum quantity of water but was usually not pure.

For further purification the cephalosporin C (sodium salt) was first converted largely into the free acid by solution in water (28 mg./ml.) and addition of Dowex 50 (H^+ form; X8) to the stirred solution until its pH fell to 2.6 (Hale, Newton & Abraham, 1961). After removal of the resin the solution was freeze-dried. The resulting powder was dissolved in 0.2 ml. of water and applied to a column (0.5 cm. $\times$ 25 cm.) of Dowex 1 (acetate form; X8; 100–200 mesh). Elution was carried out with 0.5N-acetic acid and 1 ml. fractions were collected every 5 min. The eluate was analysed by measurements of extinction at 260 μm , ninhydrin colour density (Moore & Stein, 1948) and, where [$1\text{-}^{14}\text{C}$]acetate had been added to the culture fluid, radioactivity.

Freeze-drying of the appropriate fractions (in the example given in Fig. 3, 107–170) gave an almost white powder consisting of nearly pure cephalosporin C (free acid). Solution in 2 ml. of water, titration with 0.02N- NaOH to pH 6.0 and freeze-drying gave a preparation of the sodium salt which was obtained crystalline by solution in water (concentration about 150 mg./ml.) followed by careful addition of ethanol.

For the isolation of cephalosporin C from the 16 l. and 3 l. fermentations a column of Amberlite IR-4B (100–200 mesh) was used in place of one of Amberlite XE-58. Elution from the column was carried out with pyridine acetate buffer (M with respect to pyridine), pH 5.5, instead of with ammonium acetate, and the eluate was analysed by bioassay instead of by measurement of extinction at 260 μm . The product from this column was then chromatographed on Amberlite XE-58 in pyridine acetate buffer (M with respect to pyridine), pH 5.5. The fractions of the eluate containing cephalosporin C were freeze-dried, and the residue was dissolved in the minimum amount of water and reprecipitated with acetone. The product was then dissolved in water, the pH of the solution adjusted to 7.5 with dilute NaOH and the solution freeze-dried. The resulting sodium salt was obtained crystalline as described by Newton & Abraham (1956) and the crystals were washed on the filter with 70% (v/v) ethanol.

Radioactivity measurements

Sodium [$1\text{-}^{14}\text{C}$]acetate (8.12 mc/m-mole) was obtained from The Radiochemical Centre, Amersham, Bucks. Measurements of radioactivity were carried out with a helium-filled bell-shaped Geiger-Müller counter which had a thin mica end-window. 'Infinitely thin' samples were prepared by evaporating a small known quantity (usually 30 $\mu\text{l.}$) of a dilute solution of the radioactive substance on an aluminium planchet, a disk of lens tissue being used to facilitate even spreading of the liquid. After the sample had been dried under an infrared lamp, 100 $\mu\text{l.}$ of a 0.1% solution of collodion in ether-ethanol (1:1, v/v) were applied and allowed to evaporate to prevent curling of the

lens tissue. 'Infinitely thick' samples were prepared as described by Popják (1950), except that aluminium planchets were used. The s.e. in all determinations was < 5%. Counts were compared with those from a ^{14}C -labelled polymer reference source obtained from The Radiochemical Centre. The BaCO_3 containing the ^{14}C evolved as $^{14}\text{CO}_2$ during fermentations was counted at infinite thickness.

The radioactivities of spots obtained by chromatography or electrophoresis on paper were counted directly with a tube similar to that described above, but mounted in a portable case. Sections of paper 1.5 or 3 cm. square were counted separately, a mask of X-ray film being used to isolate the area being counted. In some cases radioactive spots were first located, on the paper, by radioautography on X-ray film (Kodak Ltd., Blue Brand). The films were usually exposed for 3 weeks.

Degradation of cephalosporin C

The distribution of ^{14}C in labelled cephalosporin C was studied by degradation reactions described by Abraham & Newton (1956, 1961). Cephalosporin C was obtained from 16 l. fermentations in sufficient quantity for degradation products to be isolated (method A). Degradation products of cephalosporin C obtained from shaken-flask fermentations were studied largely by paper chromatography and electrophoresis (methods B and C). The following are examples of the procedures used.

(A) A solution of cephalosporin C (sodium salt) (300 mg.) in water (1 ml.) was brought to pH 2.65 with Dowex 50 (H^+ form; X8). The resin was separated by centrifuging and washed with a little water. The washings and supernatant combined were made 1.25N with respect to H_2SO_4 and refluxed for 1.25 hr. in a current of CO_2 -free N_2 . The CO_2 evolved was absorbed in 0.3N- $\text{Ba}(\text{OH})_2$ and the precipitated BaCO_3 washed with freshly boiled distilled water and dried (99 mg.).

The pH of the hydrolysate was adjusted to pH 2.3 with 0.3N- $\text{Ba}(\text{OH})_2$ and the precipitated BaSO_4 removed by centrifuging. The supernatant was freeze-dried, the distillate being collected in a trap cooled with ethanol-solid CO_2 . Electrometric titration of the distillate to pH 8 with 0.1N- NaOH (4.3 ml.) indicated that it contained an acid with pK_a 4.8. The titrated solution was evaporated to dryness to yield sodium acetate. The latter was converted into *p*-bromophenacyl acetate (37 mg.; m.p. 83° ; m.p. 84° when mixed with an authentic sample of m.p. 85°) by reaction with *p*-bromophenacyl bromide.

The freeze-dried residue from which acetic acid had been distilled was dissolved in water (15 ml.) and the solution extracted with ethyl acetate (15 ml.) three times. Evaporation of the extract gave a crystalline residue (38 mg.) consisting of a mixture of approximately equal amounts of compounds 1 and 2 (Abraham & Newton, 1961).

The aqueous phase separated from the ethyl acetate extract was acidified by addition of 12N-HCl (1.5 ml.) and the solution refluxed for 7 hr. The hydrolysate was evaporated to dryness and the residue chromatographed in 0.5N-acetic acid on a column (1 cm. diam. $\times$ 30 cm.) of Dowex 1 (acetate form; X10; 200–400 mesh) (Abraham & Newton, 1961). The eluate yielded crystalline *D*- α -aminoadipic acid (52 mg.).

(B) A solution of cephalosporin C (sodium salt) (180 μg .) in 1.25N-HCl (72 μl .) was heated at 100° in a sealed Pyrex-glass tube for 1 hr. A small sample of the hydrolysate

(4.9 μl .) was mixed with 0.5N- NaOH (28.3 μl .) and a sample of the mixture (28.3 μl .) was evaporated on a planchet. The radioactivity of this sample represented the total radioactivity of the cephalosporin C from which it was derived, except for that lost as CO_2 during hydrolysis.

The remainder of the hydrolysate was evaporated thoroughly to dryness. The product was dissolved in water (28.3 μl .) and a sample of the solution (4.9 μl .) evaporated on a planchet. The radioactivity of this sample, compared with that of the preceding one, gave a value for the radioactivity of the acetic acid formed on hydrolysis.

Further samples (4.9 μl .) of the hydrolysate were: (1) chromatographed on paper in butan-1-ol-acetic acid-water, which enabled compounds 1 and 2 (R_F values 0.81 and 0.85 respectively) to be distinguished from other products of hydrolysis (Abraham & Newton, 1961); (2) subjected to electrophoresis on paper in pyridine acetate buffer and then chromatographed in butan-1-ol-acetic acid-water in the perpendicular direction. By this means α -aminoadipic acid was well separated from the other known products of hydrolysis of cephalosporin C. The radioactivities of compounds 1 and 2 and of α -aminoadipic acid were counted on the chromatograms.

(C) To a solution of cephalosporin C (sodium salt) (2 mg.) in water (1 ml.) was added about 5 mg. of Raney nickel (Pavlic & Adkins, 1946) and the solution refluxed (oil bath at 130°) for 30 min. The Raney nickel was removed by centrifuging and washed with 0.25 ml. of water. To the combined supernatant and washings was added 0.2 ml. of 12N-HCl and the resulting solution was heated at 105° in a sealed tube for 18 hr. The hydrolysate was evaporated to dryness, the residue dissolved in 80 μl . of water and a sample (5 μl .) of this solution subjected to electrophoresis and chromatography on paper as described in (A). The locations of radioactive spots on the paper were revealed by radioautography. Coloration with ninhydrin revealed the presence of α -aminoadipic acid, valine, glycine and alanine (Abraham & Newton, 1956).

Isolation of α -aminoadipic acid from products other than cephalosporin C

Fractions 45–94 from the column of Amberlite XE-58 used for the purification of cephalosporin C (produced by fermentation in two shaken flasks to which [^{14}C]acetate had been added) were combined and concentrated to 3 ml. in a rotary evaporator. One vol. of 12N-HCl was added to the concentrate, the NH_4Cl which precipitated was removed by filtration, and the filtrate heated at 105° for 16 hr. in a sealed tube. The hydrolysate was evaporated to dryness and the residue was dissolved in water (1 ml.) and added to a column (1 cm. diam. $\times$ 10 cm.) of Dowex 2 (OH^- form; X10; 200–400 mesh). The column was washed with water (5 ml.) and then with 0.5N-acetic acid (about 50 ml.) until no more ninhydrin-positive material was eluted. The combined ninhydrin-positive eluates were evaporated to dryness. The residue (117 mg.) was dissolved in water (2 ml.) and the solution added to a column (1 cm. diam. $\times$ 40 cm.) of Dowex 1 (acetate form; X10; 200–400 mesh). Elution was begun with water, 2 ml. fractions being collected. Neutral and basic ninhydrin-positive material appeared in fractions 5–17 (107 mg.). After 60 fractions had been collected elution was continued with 0.5N-acetic acid. α -Aminoadipic acid appeared in fractions 115–125, glutamic acid in fractions 134–144 and aspartic acid in

fractions 202–227. Fractions 115–125 were combined and evaporated to dryness *in vacuo*. The residue was dissolved in water (2 ml.) and the total amount of α -amino adipic acid in the solution (0.706 mg.) was determined by the photometric ninhydrin method (Moore & Stein, 1948). Samples (28 μ l.) of this solution were used for counting at infinite thickness.

Degradation of α -amino-[14 C]adipic acid

In two experiments, C-1 of α -amino-[14 C]adipic acid was obtained in the form of CO_2 by the ninhydrin- CO_2 reaction of Van Slyke, Dillon, MacFadyen & Hamilton (1941). The CO_2 was absorbed in 0.3N-Ba(OH) $_2$ to give BaCO_3 . In the first experiment, 50 mg. of α -amino adipic acid yielded 54 mg. of BaCO_3 which was counted at infinite thickness. In the second experiment, 9 mg. of α -amino adipic acid yielded 9.6 mg. of BaCO_3 which was counted at infinite thickness.

One sample of α -amino-[14 C]adipic acid was degraded by the Schmidt reaction, under conditions similar to those used by Adamson (1939) and by Mosbach, Phares & Carson (1951), to yield C-6 as CO_2 and the remaining carbon atoms in the form of ornithine. α -Amino adipic acid (2 mg.) was weighed into a Pyrex-glass reaction tube (1 cm. diam. $\times$ 6 cm.) drawn to a tip at the bottom. The tube was fitted at the top with a ground-glass joint which carried a narrower entrance tube terminating 1 cm. from the bottom, and with a side-arm exit through which it was connected by rubber tubing to two absorption vessels in series, each of which contained 2 ml. of 0.1N-NaOH (carbonate free). Air was removed from the apparatus by a stream of N_2 , and the entrance and exit tubes were then closed. The reaction vessel was opened at the glass joint and a solution of NaN_3 (0.8 mg.) in conc. H_2SO_4 (10 μ l.) was added to the α -amino adipic acid. The vessel was reclosed and its tip immersed in water at 45° for 4 hr. The CO_2 formed was then swept into the NaOH by a stream of N_2 . To the NaOH in each absorption vessel was added NH_4Cl (8 mg.), followed by BaCl_2 (30 mg.) in 1 ml. of water (Calvin, Heidelberger, Reid, Tolbert & Yankwich, 1949). The precipitated BaCO_3 (1.0 mg.) was filtered off and counted at infinite thickness.

The mixture remaining in the reaction tube was diluted with water (0.4 ml.) and the solution added to a column (1 cm. diam. $\times$ 5 cm.) of Dowex 1 (acetate form; X 10; 200–400 mesh). The column was washed with water and 0.5 ml. fractions were collected. Ornithine appeared in fractions 2–4 and was identified by paper chromatography and electrophoresis. The amount of ornithine in the combined fractions (0.65 mg.) was determined by the photometric ninhydrin reaction (Moore & Stein, 1948) and its radioactivity was counted at infinite thickness.

RESULTS

Production and purification of cephalosporin C labelled with ^{14}C from [1- ^{14}C]acetate

With fermentations in shaken flasks, cephalosporin C began to be detectable by bioassay in the culture fluid after about 24 hr. Its concentration increased rapidly during the next 24 hr. and then slowly to between 140 and 200 $\mu\text{g./ml.}$, 70 hr. after inoculation, when it was harvested (Fig. 1). The rapid phase of cephalosporin C produc-

tion was accompanied by a fall in the pH of the medium from 7.0 to 6.1 and the subsequent slower phase with a rise in pH to about 7.6. The concentration of volatile weak acid in the culture fluid decreased slowly for about 12 hr. and then more rapidly, falling to less than 10% of its original value in about 24 hr. (Fig. 1). Paper chromatography and electrophoresis indicated that this acid consisted almost entirely of acetic acid. Formic acid was also detected, but its concentration did not exceed 5% of the total volatile organic acid originally present. With fermentations to which [1- ^{14}C]acetate had been added about 30% of the total radioactivity was finally recovered in the respiratory carbon dioxide (as barium carbonate) and about 30% in the mycelium.

Analysis of the eluate from the column of Amberlite XE-58 by measurements of extinction at 260 $\text{m}\mu$ revealed three main bands (Fig. 2). The first to be eluted, which was composite (A), contained peptides and amino acids which showed no net charge when subjected to electrophoresis on paper at pH 4.5. The second band (B) contained acidic ninhydrin-positive substances among which should have been any penillic acid formed during the inactivation of penicillin N in the culture fluid, since this penillic acid was found in earlier work to be eluted in a similar position (Newton & Abraham, 1956). The material in bands A and B showed no absorption maximum in ultraviolet light but an increasing extinction from 300 to 220 $\text{m}\mu$. The third band (C) contained cephalosporin C ($\lambda_{\text{max.}}$ 260 $\text{m}\mu$) which could be induced to crystallize in the form of its sodium salt but which was only about 50% pure.

When [1- ^{14}C]acetate had been added to the fermentations 24 or 48 hr. after inoculation, radio-

Fig. 1. Decrease in the concentration of volatile weak acid and formation of cephalosporin C in fermentations in shaken flasks. •, Volatile weak acid; x, cephalosporin C. Determinations were made on pooled samples from 10 flasks.

activity appeared in bands A, B and C, and with A and B its intensity varied roughly in parallel with extinction. Qualitative studies of the composition of the material in bands A and B respectively were made by hydrolysis of a sample of the material in 6N-HCl at 105° for 16 hr., followed by electrophoresis of the hydrolysate on paper at pH 4.5 and chromatography on paper in butan-1-ol-acetic acid-water. Amino acids were detected with ninhydrin and radioactive spots by radioautography. Band A appeared to yield at least ten common amino acids, among which glutamic acid, aspartic acids, alanine and leucine (or isoleucine, or both), but not lysine, showed detectable radioactivity. Band B yielded a similar mixture of common α -amino acids and also a small proportion of α -amino adipic acid. A comparison of radioautographs with ninhydrin colours on the corresponding chromatograms showed that the α -amino adipic acid was

strongly radioactive, but that the radioactivity of the glutamic acid was relatively weak. The radioactivity of the aspartic acid was scarcely detectable under the conditions used.

With one fermentation to which [1-¹⁴C]acetate had been added 48 hr. after inoculation the α -amino adipic acid from band B was isolated and degraded by the Schmidt reaction to give radioactive carbon dioxide and ornithine. By this procedure it was shown that about 36% of the total radioactivity of the α -amino adipic acid was present in C-6. Aspartic acid isolated from band B in the same experiment showed only 1.6% of the molar radioactivity of the α -amino adipic acid. In addition to the amino acids coloured with ninhydrin a highly radioactive ninhydrin-negative compound with R_f slightly greater than that of proline and no net charge at pH 4.5 was present in the hydrolysate from band B.

With band C the curve obtained by plotting radioactivity against the volume of eluate did not coincide with that obtained by plotting extinction. This was accounted for by the presence of radioactive material which did not absorb at 260 m μ and which appeared in the eluate slightly before cephalosporin C. Most of the contaminating material contained no basic function and appeared in the effluent when the cephalosporin C was adsorbed on a column of Dowex 50. In one experiment ([1-¹⁴C]-acetate added 48 hr. after inoculation) the radioactivity of the effluent comprised about 70% of the total radioactivity added to the column. Paper electrophoresis and chromatography, followed by radioautography, showed that the effluent contained at least five radioactive compounds. One of these, representing some 30% of the total radioactivity of the mixture, could be detected on paper by the chlorination procedure of Rydon & Smith (1952), and corresponded in behaviour to 6-oxo-piperidine-2-carboxylic acid. On hydrolysis with 2N-hydrochloric acid at 105° for 16 hr., the mixture yielded at least 10 different amino acids, of which only α -amino adipic acid showed a radioactivity high enough to be detected on paper under the conditions used for chromatography and electrophoresis. A considerable proportion of the total radioactivity in the mixture failed to appear in the residue obtained on evaporation of the acid hydrolysate.

Chromatography on Dowex 1 of the material eluted from Dowex 50 separated cephalosporin C from a relatively small amount of ninhydrin-positive material which showed no selective absorption in ultraviolet light and a low radioactivity (Fig. 3). A major component of this material migrated to the same position as cephalosporin C on paper electrophoresis at pH 4.5 but showed a lower R_f (0.1) than the latter when chromato-

Fig. 2. Purification of radioactive cephalosporin C by chromatography on Amberlite XE-58. ●, Extinction at 260 m μ ; ○, radioactivity. The cephalosporin C had been produced by fermentations in two shaken flasks to which [1-¹⁴C]acetate had been added 48 hr. after inoculation. Fractions 1-37 (band A), 45-94 (band B) and 95-170 (band C) were combined for further study.

Fig. 3. Purification of radioactive cephalosporin C by chromatography on Dowex 1 X8. ●, Extinction at 260 m μ ; ○, radioactivity; x, ninhydrin colour density. The material added to the column was derived from fractions 95-170 of the eluate shown in Fig. 2.

Table 1. *Incorporation of [1-¹⁴C]acetate into cephalosporin C*

Fermentations with 200 ml. of medium were in shaken flasks and the remainder in vessels equipped for stirring and aeration as described in the text. Solutions of sodium [1-¹⁴C]acetate were sterilized by autoclaving before addition at various times after inoculation. The concentration of acetate in medium B1 was initially twice that in B2. The concentrations of total acetate in the media at the times of addition of [1-¹⁴C]acetate have been assumed to be identical with those determined with similar fermentations to which no isotope had been added. For the procedures used for the estimation of acetate and isolation of cephalosporin C, see text.

Fermentation no.	Medium	Vol. (l.)	Amount of [1- ¹⁴ C]acetate added (mc)	Time (t) after which [1- ¹⁴ C]acetate was added (hr.)	Assumed concn. of total acetate in culture fluid at time t (mM)	Specific radioactivity (μC/m-mole)		Apparent dilution of molar radioactivity (A/B)
						Assumed total acetate at time t (A)	Cephalosporin C isolated (B)	
1	B1	3	1.0	16	19.0	17.6	5.76	3.1
2	B1	16	0.1	24	14.4	0.45	0.15	3.0
3	B2	0.2	0.2	24	1.3	810	2.28	350
4	B2	0.2	0.2	48	0.1*	8120	14.6	555

* This is a minimum value based on the assumption that unlabelled acetate had completely disappeared from the culture medium when labelled acetate was added. The method of estimation was capable of showing only that the concentration of unlabelled acetate fell to less than 0.2 mM. The corresponding value for dilution of molar radioactivity is therefore a maximum value.

graphed on paper in butan-1-ol-acetic acid-water. On acid hydrolysis it yielded a mixture of ninhydrin-positive products which appeared to consist mainly of aspartic acid together with a small proportion of glycine and a smaller proportion of α-amino adipic acid. Radioautography after paper chromatography and electrophoresis indicated that the α-amino adipic acid and aspartic acid were radioactive, but that the former had a much higher molar radioactivity than the latter.

Measurements of extinction at 260 mμ were used to obtain maximum values for the amounts of cephalosporin C in the products from different stages of the isolation process applied to 200 ml. of culture fluid. They indicated that the material eluted from Dowex 50 X8 contained up to 80 % of the cephalosporin C in the culture fluid and was about 50 % pure. This material would crystallize as a sodium salt but the yields of pure cephalosporin C that could be obtained from it by recrystallization were poor. After chromatography on Dowex 1 X8 the product was about 70 % pure and the overall recovery about 25 %. Material of this purity could be recrystallized to give virtually pure cephalosporin C (sodium salt) in yields of over 50 %.

Table 1 shows the molar radioactivities of samples of cephalosporin C (sodium salt) isolated from fermentations to which [1-¹⁴C]acetate had been added at different times. In relation to the amounts of isotope added/unit volume of culture medium the molar activities of the different samples were of the same order of magnitude. But the apparent molar dilution of the isotope in cephalosporin C, calculated on the basis of the molar radioactivity of the total acetate assumed to be present

in the medium when the labelled acetate was added, showed considerable variation. The dilution was much greater in cephalosporin C obtained from fermentations in shaken flasks with medium B2 than it was in cephalosporin C obtained from the larger-scale fermentations with medium B1, even when the addition of [1-¹⁴C]acetate had been made to both types of fermentation at the same time after inoculation.

Distribution of ¹⁴C in labelled cephalosporin C

Acid hydrolysis of cephalosporin C labelled with ¹⁴C from [1-¹⁴C]acetate yielded the following degradation products: α-amino adipic acid from the δ-(α-amino adipoyl) side chain; acetic acid from the acetoxy group; carbon dioxide from the β-lactam (C-8); compounds 1 and 2 from C-2, C-3, C-10, C-4 and C-9 (Abraham & Newton, 1961). Table 2 gives the molar radioactivities of these products expressed as percentages of the molar radioactivities of the samples of cephalosporin C from which they were obtained. The results show that 86-92 % of the radioactivity of cephalosporin C was present in the α-amino adipoyl and acetoxy groups. The carbon dioxide accounted for less than 2 % and the compounds 1 and 2 for less than 4 %. About 43 % of the radioactivity of the α-amino adipic acid residue was found in the carbon atom of the α-carboxyl group (C-1) when the cephalosporin C concerned was isolated from a fermentation (in medium B1) to which [1-¹⁴C]acetate had been added after 16 hr., and 67 % when the labelled acetate had been added after 24 hr. The former value, which was obtained from the degradation of a relatively small amount of α-amino adipic acid, is likely to be less accurate than the latter.

Table 2. *Distribution of radioactivity in ^{14}C -labelled cephalosporin C*

The fermentations are numbered as in Table 2. For details of the degradative procedures see text. With cephalosporin C from fermentations 1 and 2 degradation products were isolated. With cephalosporin C from fermentations 3 and 4 degradation products were analysed by paper electrophoresis and chromatography. —, Not determined. Compounds 1 and 2 were not separated from each other and were assumed to be present in equimolar amounts.

Cephalosporin C from fermentation no.	Molar radioactivity (as % of that of cephalosporin C)				
	CO_2	Compounds 1 and 2	Acetic acid	α -Amino adipic acid	CO_2 from C-1 of α -amino adipic acid
1	1.5	4	26	66	28
2	1	2	34	52	35
3	—	4	34	55	—
4	—	—	43	49*	—

* This value may be low. A value of 52% would have been obtained on the assumption that the molar radioactivity of the α -amino adipic acid from cephalosporin C was identical with that found for a sample of α -amino adipic acid isolated from material in band B (Fig. 2).

Treatment of labelled cephalosporin C with Raney nickel and hydrolysis of the product with acid yielded material which showed ninhydrin-positive spots, after paper electrophoresis and chromatography, that corresponded to α -amino adipic acid, alanine (from C-6, C-7 and C-8), glycine (from C-7 and C-8) and valine (from C-2, C-3, C-10, C-4 and C-9) respectively. Radioautography revealed radioactivity in the spot corresponding to α -amino adipic acid, but not in the spots corresponding to alanine, glycine or valine. It also revealed radioactivity in a spot which was not coloured by ninhydrin but which could be detected by the chlorination procedure of Rydon & Smith (1952). This degradation product of cephalosporin C showed no net charge on paper electrophoresis at pH 4.5 and R_{valine} 0.78 on paper chromatography in butan-1-ol-acetic acid-water.

DISCUSSION

The procedure described above enabled cephalosporin C to be isolated from small volumes of culture fluid in the form of a sodium salt which was more than 70% pure and in an overall yield of about 25%. The product thus obtained yielded substantially pure cephalosporin C (sodium salt) on crystallization. Attempts to shorten the procedure for obtaining a product with a high degree of purity were not successful in the present work, but it is possible that a simpler process would be satisfactory for fermentations with other culture media.

When $[1-^{14}\text{C}]$ acetate was added to fermentations at various times after inoculation, ^{14}C was incorporated into cephalosporin C. Further results would be needed to assess the significance of the differences in the apparent molar dilution of radioactivity in samples of cephalosporin C isolated from different experiments. But the high value for the

molar dilution when the addition of $[1-^{14}\text{C}]$ acetate to a fermentation in shaken flasks with medium B2 was made 24 hr. after inoculation might be associated with a rapid metabolism of exogenous acetate which resulted in the virtual disappearance of the latter before a significant proportion of the total cephalosporin C had been formed (Fig. 1); with a fermentation in medium B1 the concentration of acetate was initially twice that in medium B2 and decreased more slowly (Table 1). When $[1-^{14}\text{C}]$ acetate was added to a fermentation 48 hr. after inoculation, most of the total cephalosporin C had been formed before the addition was made. The molar dilution of radioactivity in the cephalosporin C that was synthesized after the addition of isotope should be about one-tenth that shown in Table 1.

Degradation of samples of cephalosporin C produced in the presence of $[1-^{14}\text{C}]$ acetate indicated that in each case acetate was incorporated into the acetoxy group and the δ -(α -amino adipoyl) group of the antibiotic, but not to a comparable extent into the remainder of the molecule. The α -amino adipoyl group of the cephalosporin C from three, at least, of four fermentations showed a higher molar radioactivity than the acetoxy group, although there was some indication of a relative increase in the radioactivity of the acetoxy group with an increase in the time from the beginning of the fermentation at which $[1-^{14}\text{C}]$ acetate had been added.

Where the most reliable determinations were made (fermentation 2, Table 2), the radioactivity of C-1 of the α -amino adipic acid residue from cephalosporin C was approximately the same as that of the labelled carbon of the acetoxy group. This is consistent with the view that C-1 of the α -amino adipic acid was derived directly from acetate. About 67% of the total radioactivity of the α -amino adipic acid was present in C-1. In conjunction with the finding that a sample of α -amino adipic acid obtained by hydrolysis of

material (band B, Fig. 2) that had been separated from cephalosporin C during the process of purification (fermentation 4, Table 2) contained about 36 % of its activity in C-6, this suggests that the isotope of $[1-^{14}\text{C}]$ acetate is incorporated mainly into C-1 and C-6 of α -aminoadipic acid.

These results are consistent with a pathway suggested by Strassman & Weinhouse (1953) for the biosynthesis of α -aminoadipic acid in *Torulopsis utilis*, according to which acetyl-coenzyme A condenses with α -oxoglutarate to yield α -oxoadipate by reactions analogous to those of the citric acid cycle involved in the formation of α -oxoglutarate from acetyl-coenzyme A and oxaloacetate. Since oxaloacetate formed from pyruvate and $[^{14}\text{C}]$ carbon dioxide in liver is known to yield α -oxoglutarate which has ^{14}C only in the carboxyl group adjacent to its ketonic group (Potter & Heidelberger, 1949), α -oxoglutarate formed from $[1-^{14}\text{C}]$ acetate and oxaloacetate would be expected to be labelled as follows:

Further evidence for this type of labelling comes from the finding that glutamic acid formed by *Escherichia coli* in the presence of $[1-^{14}\text{C}]$ acetate contains more than 80 % of its total ^{14}C in C-5 (Roberts, Cowie, Abelson, Bolton & Britten, 1955). α -Oxoglutarate with ^{14}C in its γ -carboxyl group should condense with $[1-^{14}\text{C}]$ acetate to yield α -oxoadipic acid which would give α -aminoadipic acid with ^{14}C in both C-1 and C-6:

The degree of labelling of C-6 of the α -aminoadipic acid would depend on the extent to which the α -oxoglutarate that condensed with $[1-^{14}\text{C}]$ acetate had itself been formed by condensation of $[1-^{14}\text{C}]$ acetate with oxaloacetate. Unlabelled α -oxoglutarate might have been formed, for example, from unlabelled glutamic acid present in the original culture medium, or from glutamic acid synthesized by the organism before the isotope had been added. The molar radioactivity of the glutamic acid formed on hydrolysis of some of the by-products encountered during the purification of cephalosporin C appeared to be considerably less than half that of the α -aminoadipic acid from the cephalosporin C and from other products obtained in the same experiments.

The present results do not exclude an alternative pathway for the biosynthesis of α -aminoadipic acid, thought to be less likely by Strassman & Weinhouse (1953), according to which acetate condenses with succinyl-coenzyme A formed from α -ketoglutarate. However, the formation from $[1-^{14}\text{C}]$ acetate of α -aminoadipic acid labelled exclusively in C-1 and C-6 would not be consistent with a pathway involving the condensation of acetate with succinate produced in the glyoxylic acid cycle (Kornberg & Krebs, 1957). In this case ^{14}C would be distributed between both carboxyl carbons of the succinic acid and would be expected to appear in C-1, C-3 and C-6 of α -aminoadipic acid.

Some α -aminoadipic acid was presumably added to the culture media used for the fermentations described here, since this amino acid has been found in corn-steep liquor. However, the amount of α -aminoadipic acid added is likely to have been very much less than that synthesized by the *Cephalosporium* sp. The total concentration of α -aminoadipic acid in corn-steep liquor was reported by Windsor (1951) to be 0.13 %. This would correspond to a molar concentration of α -aminoadipic acid in the culture media which was less than 4 % of that of the cephalosporin C formed during the fermentations. The finding that the radioactivity of C-1 of the α -aminoadipic acid residue in cephalosporin C was similar to that of the labelled carbon of the acetoxy group suggests that acetate was incorporated into all or most of the α -aminoadipic acid synthesized during the fermentations. The dilution of radioactivity in the α -aminoadipic acid synthesized after the addition of isotope would then reflect the dilution of exogenous $[1-^{14}\text{C}]$ acetate with unlabelled acetate produced by the *Cephalosporium* sp. from carbohydrate and other substrates.

Of the uncharacterized products from the fermentations which yielded α -aminoadipic acid on acid hydrolysis, one may have been penicillin N penicillic acid. Although free α -aminoadipic acid was not detected among the products separated during the purification of cephalosporin C, one of these products was free 6-oxopiperidine-2-carboxylic acid with a molar radioactivity similar to that of the α -aminoadipic acid residue in cephalosporin C formed in the same fermentations.

SUMMARY

1. Cephalosporin C was produced by fermentations of a *Cephalosporium* sp. in shaken flasks containing a complex culture medium. It was isolated from relatively small volumes of culture fluid by chromatography on ion-exchange resins.

2. Cephalosporin C which had been formed in the presence of $[1-^{14}\text{C}]$ acetate was radioactive.

About 90 % of its radioactivity was localized in the α -aminoadipoyl and acetoxy groups of the molecule.

3. The radioactivity of C-1 of the D- α -amino-adipic acid from labelled cephalosporin C was similar to that of the labelled carbon of the acetic acid. Evidence was obtained that the radioactivity of C-1 and C-6 of the α -aminoadipic acid accounted for most of the total radioactivity of the molecule.

4. The results are consistent with the hypothesis that the carbon skeleton of α -aminoadipic acid is formed in the *Cephalosporium* sp. from acetyl-coenzyme A and the succinate moiety of α -oxoglutarate.

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