Schmidt reaction to ornithine. The results showed that almost all the ^{14}C in the α-aminoadipic acid was localized in C-6.

3. A small proportion of the radioactivity of the cephalosporin C was found in compounds 1 and 2, derived from the C_5 fragment of the molecule which yields valine on hydrogenolysis. Penicillaminic acid with a radioactivity similar to that of the C_5 fragment of cephalosporin C was isolated from a fraction which probably contained the penillic acid from penicillin N.

4. Glutamic acid and aspartic acid, isolated from other products of the fermentation, showed a molar radioactivity which was considerably lower than that of the α-aminoadipic acid from cephalosporin C. 6-Oxopiperidine-2-carboxylic acid, which was separated from cephalosporin C during the process of purification, showed a molar radioactivity which was 36% of that of the α-aminoadipic acid from cephalosporin C.

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REFERENCES

Abraham, E. P. & Newton, G. G. F. (1961). *Biochem. J.* **79**, 377.
Abraham, E. P. & Trown, P. W. (1963). *Biochem. J.* **86**, 271.
Arnstein, H. R. V. & Grant, P. T. (1954). *Biochem. J.* **57**, 353.
Gross, D. (1958). *Nature, Lond.*, **181**, 264.
Gross, D. (1961). *J. Chromat.* **5**, 194.
Hayaishi, O., Shimazono, H., Katagiri, M. & Saito, Y. (1956). *J. Amer. chem. Soc.* **78**, 5126.
Hirs, C. H. W. (1956). *J. biol. Chem.* **219**, 611.
Kornberg, H. L. & Krebs, H. A. (1957). *Nature, Lond.*, **179**, 988.
Lewis, K. F. & Weinhouse, S. (1951). *J. Amer. chem. Soc.* **73**, 2906.
Moore, S. & Stein, W. H. (1948). *J. biol. Chem.* **176**, 367.
Rydon, H. N. & Smith, P. W. G. (1952). *Nature, Lond.*, **169**, 922.
Srere, P. A. (1959). *J. biol. Chem.* **234**, 2544.
Srere, P. A. (1961). *J. biol. Chem.* **236**, 50.
Strassman, M., Thomas, A. J. & Weinhouse, S. (1955). *J. Amer. chem. Soc.* **77**, 1261.
Trown, P. W., Abraham, E. P., Newton, G. G. F., Hale, C. W. & Miller, G. A. (1962). *Biochem. J.* **84**, 157.
Trown, P. W., Smith, B. & Abraham, E. P. (1963). *Biochem. J.* **86**, 284.

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Biosynthesis of Cephalosporin C from Amino Acids

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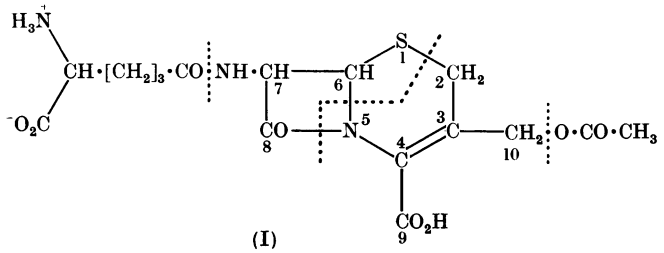
The structure of cephalosporin C can be dissected into residues of D-α-aminoadipic acid and L-cysteine, a C_5 fragment which yields DL-valine on hydrogenolysis, and an acetoxy group, as shown by the broken lines in (I) (Abraham & Newton, 1961). Evidence has been obtained for the biosynthesis of the carbon skeleton of the α-aminoadipic acid residue from acetate and α-oxoglutarate (Trown, Abraham, Newton, Hale & Miller, 1962; Trown, Sharp & Abraham, 1963).

The experiments described in the present paper were done to ascertain whether exogenous α-aminoadipic acid acts as a precursor of the side chain of cephalosporin C and whether cystine and



(I)

valine are incorporated into the β-lactam–dihydrothiazine ring system, as they are into the β-lactam–thiazolidine ring system of benzylpenicillin (Arnstein, 1957).

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 when otherwise stated, electrophoresis, on Whatman no. 1 paper, was carried out in pyridine–acetate buffer (0·05 M with respect to acetate), pH 4·5, or in $(NH_4)_2CO_3$ buffer (7·9 g./l.), pH 8·9, as described by Abraham & Trown (1963).

Amino acids were detected on paper by reaction with ninhydrin. Compounds 1 and 2 were detected by their absorption of ultraviolet light (Abraham & Newton, 1961). Acetic acid was detected after electrophoresis in $(NH_4)_2CO_3$ buffer by spraying the paper with a solution of bromophenol blue (0·025%) in acetone–water (9:1, v/v) containing citric acid (0·1%, w/v), as described by Gross (1958). Cephalosporidine (Abraham & Trown, 1963) and 6-oxopiperidine-2-carboxylic acid, which showed no ninhydrin reaction, were detected on paper by a modification of the method of Rydon & Smith (1952) as described by Trown *et al.* (1962).

6-Oxopiperidine-2-carboxylic acid migrated 3 times as far as α-aminoadipic acid towards the anode on electrophoresis at pH 4·5. The extent to which α-amino[2-^{14}C]adipic acid was converted into 6-oxo[2-^{14}C]piperidine-2-carboxylic acid when an aqueous solution of its sodium salt was autoclaved (10 lb./in.^{2} for 10 min.) was determined from the counts given by spots corresponding to the two compounds after the latter had been separated by electrophoresis on paper.

Semi-quantitative determinations of the amounts of free valine in the culture fluid (medium B1 of Trown *et al.* 1962) at different stages of the fermentation were made as described by Swallow & Abraham (1958) after separation of the valine from other amino acids by paper chromatography in butan-1-ol–acetic acid–water (4:1:4, by vol.). Samples (5 ml.) of the culture fluid were mixed with 1 ml. of 10% (w/v) trichloroacetic acid and the precipitate was removed by centrifuging. The supernatant was desalted by adsorption on a column (1 cm. diam. × 2 cm.) of Dowex 50 (200–400 mesh; H^+ form; X8) and elution with dilute NH_3 until the pH rose to 8. A sample (2·7 mg.) of the residue was oxidized with performic acid (Hirs, 1956) to convert methionine (which has the same R_F as valine in the system used for paper chromatography) into its sulphone. The oxidized product was dissolved in water (0·2 ml.) and a sample (5 μl.) of this solution applied to the paper chromatogram.

Fermentation methods

Maintenance of the culture (*Cephalosporium* sp. C.M.I. 49 137, mutant 8650), preparation of the inoculum, fermentations in shaken flasks (each flask containing 100 ml. of the complex medium B1 or B3), and bioassay of the antibiotic were all carried out as described by Trown *et al.* (1962, 1963).

Isolation of ^{14}C-labelled cephalosporin C

The procedure for isolation of crystalline cephalosporin C (sodium salt) (from the contents of two shaken flasks) was that described by Trown *et al.* (1962), except that the antibiotic was usually of high enough specific radioactivity to allow dilution (about threefold) with non-labelled crystalline cephalosporin C (sodium salt) before the final crystallization from aqueous ethanol. The degree of dilution was determined by measurements of the extinction at 260 mμ, at which cephalosporin C shows $E_{max.}$. The amount of final product obtained enabled more extensive degradations to be undertaken than was the case with the cephalosporin C isolated by Trown *et al.* (1962) from fermentations in shaken flasks.

Radioactive compounds and radioactivity measurements

DL-[1-^{14}C]Valine (4·76 mc/m-mole), a mixture (approx. 1:1) of DL-[3-^{14}C]cystine and *meso*-[3-^{14}C]cystine (1·83 mc/m-mole), and diethyl acetamido[2-^{14}C]malonate (1·0 mc/m-mole) were obtained from The Radiochemical Centre, Amersham, Bucks. Radioactivity measurements and radioautographs were made as described by Trown *et al.* (1962).

Synthesis of DL-α-*amino*[2-^{14}C]*adipic acid.* This amino acid was synthesized from γ-bromobutyronitrile and diethyl acetamido[2-^{14}C]malonate as follows: Unlabelled diethyl acetamidomalonate (21 mg.; 0·1 m-mole) was added to the radioactive compound (21 mg.) and the mixture added to 0·3 m-mole of NaH (14·4 mg. of a 50% dispersion in oil) (L. Light and Co. Ltd., Colnbrook, Bucks.) (Shapira, Shapira & Dittmer, 1953) in a flat-bottomed Pyrex test-tube (1 cm. diam.). γ-Bromobutyronitrile (L. Light and Co. Ltd.) (44·5 mg.; 0·3 m-mole) and redistilled toluene (0·2 ml.) were then added, together with a small magnetic stirrer. The tube was sealed, and the mixture stirred and heated at 110° for 40 hr. The tube was then cooled and opened, the toluene evaporated, and the reaction product transferred to another Pyrex tube with 6 N-HCl. The latter tube was sealed and heated at 110° for 16 hr. The contents of the tube were then evaporated to dryness and the residue was dissolved in water (2 ml.) and applied to a column (1 cm. diam. × 40 cm.) of Dowex 1 (100–200 mesh; acetate form; X10) previously washed with 2 bed-volumes of water. Water was passed through the column at 50 ml./hr., fractions being collected at 6 min. intervals. After 22 fractions (110 ml.) had been collected, and all the neutral and basic impurities had been removed from the column, 0·5 N-acetic acid was applied at the same rate. α-Aminoadipic acid appeared in fractions 52–60, which were combined and evaporated *in vacuo* to give a crystalline product (15·2 mg.; yield 47%). This product was indistinguishable from authentic α-aminoadipic acid when subjected to electrophoresis on paper in pyridine–acetate buffer (0·05 M with respect to acetate), pH 4·5, and chromatography in butan-1-ol–acetic acid–water (4:1:4, by vol.). Its purity, as estimated by colour yield in the photometric ninhydrin procedure of Moore & Stein (1948), was 95%. Its specific radioactivity was found to be 0·51 mc/m-mole by comparison with a ^{14}C-labelled polymer reference source obtained from The Radiochemical Centre. The product was used as a solution of the sodium salt, obtained by the addition of water (2 ml.) and adjustment of the pH to 7·0 with 4 N-NaOH.

Degradation of ^{14}C-labelled cephalosporin C

Qualitative studies of the distribution of radioactivity among the products formed from [^{14}C]cephalosporin C on

hydrolysis with 1·25 N-HCl at 105° for 1 hr., or on hydrogenolysis with Raney nickel followed by acid hydrolysis, were made by paper electrophoresis and chromatography, followed by radioautography, as described by Trown *et al.* (1962). In some cases, the radioactivities of spots corresponding to degradation products, including compounds 1 and 2 (Abraham & Newton, 1961), were counted directly on the papers. Quantitative estimations of the molar radioactivities of acetate, α-aminoadipic acid and compounds 1 and 2 from approximately 2 mg. quantities of cephalosporin C were made as described by Trown *et al.* (1963).

Determination of radioactivity in C-6 *plus* C-7. In one case cephalosporin C was degraded to yield C-6 and C-7 (two of the carbon atoms in its β-lactam ring) as C-1 and C-2 respectively of glycine. δ-Amino-δ-carboxyvalerylglycine was isolated from cephalosporin C by a modification of the process described by Jeffery, Abraham & Newton (1960) and hydrolysed to give α-aminoadipic acid and glycine.

A solution of the sodium salt of cephalosporin C (10 mg.) in 0·1 N-HCl (0·5 ml.) was heated in a sealed Pyrex tube at 100° for 18 min. The hydrolysate was evaporated to dryness in a vacuum desiccator in the presence of solid KOH. The residue was dissolved in water (0·2 ml.) and applied to a column (0·5 cm. diam. × 2 cm.) of Dowex 1 (100–200 mesh; acetate form; X10) which had been previously washed with water. On elution with water, neutral and basic substances passed rapidly through the column and were collected in 3 ml. of effluent. The effluent was treated directly with Ag_2O (freshly precipitated from a solution of 20 mg. of $AgNO_3$) at 80° for 1·5 hr. The product was filtered, the filtrate brought to pH 3 with 2 N-HCl, and the precipitated AgCl removed by centrifuging. The supernatant was evaporated to dryness, and the residue redissolved in water (0·1 ml.) and applied to a second column (0·5 cm. diam. × 2 cm.) of Dowex 1 (100–200 mesh; acetate form; X10). Neutral material was eluted with water (6 ml.). Elution was then continued with 0·5 N-acetic acid, 1 ml. fractions being collected. Fractions 1–3 were ninhydrin-positive and were combined and evaporated to dryness to yield a white solid (0·53 mg.). This solid was dissolved in water (56·6 μl.) and samples (4·6 μl.) of the solution were subjected to electrophoresis on paper in pyridine–acetate buffer (0·05 M with respect to acetate), pH 4·5, and chromatography on paper in butan-1-ol–acetic acid–water (4:1:4, by vol.). It was thereby shown to consist of a mixture of α-aminoadipic acid and a compound which was indistinguishable from a synthetic sample of δ-amino-δ-carboxyvalerylglycine (Abraham & Newton, 1954).

The remainder of the solution was made 1 N with respect to HCl and heated in a sealed tube at 110° for 16 hr. The contents of the tube were evaporated to dryness and the residue was dissolved in water (56·6 μl.). A sample (4·6 μl.) of this solution was subjected to electrophoresis on paper in pyridine–acetate buffer (0·05 M with respect to acetate), pH 4·5, followed by chromatography in butan-1-ol–acetic acid–water (4:1:4, by vol.) in the perpendicular direction. Coloration with ninhydrin revealed the presence of only two spots, corresponding to α-aminoadipic acid and glycine respectively.

The remainder of the hydrolysate was applied to a column (0·5 cm. diam. × 2 cm.) of Dowex 1 (100–200 mesh; acetate form; X10) previously well washed with water. Elution with water (2 ml.) gave glycine; subsequent elution with 0·5 N-acetic acid (2·5 ml.) gave α-aminoadipic acid. These two amino acids were obtained as white crystalline compounds by evaporation of the eluates to dryness. The glycine was dissolved in water (200 μl.) and the α-aminoadipic acid in 0·1 N-HCl (500 μl.), and their respective concentrations were measured by the photometric ninhydrin method of Moore & Stein (1948). Samples (28·3 μl.) were evaporated on planchets for radioactivity measurements.

Determination of radioactivity in C-8. Unlabelled crystalline cephalosporin C (sodium salt) (10 mg.) was added to a sample (0·9 mg.) of the radioactive cephalosporin C (sodium salt) obtained from Expt. 2 (Table 1) and the mixture dissolved in 1·25 N-H_2SO_4. The solution was refluxed for 2·5 hr. in a current of CO_2-free N_2. The CO_2 evolved was absorbed in two traps each containing 2 ml. of 0·3 N-$Ba(OH)_2$. The precipitated $BaCO_3$ was separated by centrifuging, washed with CO_2-free water, dried and weighed (3·8 mg.). A sample (2·23 mg.) of this $BaCO_3$ was applied to a planchet as a slurry in ethanol (100 μl.) for counting. A correction for self-absorption was made from data given by Calvin, Heidelberger, Reid, Tolbert & Yankwich (1949).

Isolation of other substances from culture fluids and mycelium

Material in bands A *and* B. During the purification of cephalosporin C by chromatography on Amberlite XE-58 in ammonium acetate buffer (Trown *et al.* 1962) two main bands of material (*A* and *B* respectively) appeared before cephalosporin C in the eluate. The fractions containing each of these bands were combined and the two solutions evaporated to dryness. Each residue, which still contained some ammonium acetate, was dissolved in water (2 ml.) and applied to a column (2 cm. diam. × 10 cm.) of Dowex 50 (100–200 mesh; H^+ form; X4). The column was washed with water until the pH of the effluent (which contained taurine in the case of band *A*) rose to 5. Other ninhydrin-positive substances were eluted by passing 4 N-NH_3 through the column until the pH rose to 8·0. The eluate was freeze-dried and the residue used in subsequent studies of the material in the band concerned.

α-*Aminoadipic acid.* This amino acid was isolated from material in band *B*, which was obtained as described above, from a fermentation to which α-amino[2-^{14}C]adipic acid had been added. The material (120 mg.) was dissolved in water (2 ml.) and added to a column (1 cm. diam. × 40 cm.) of Dowex 1 (200–400 mesh; acetate form; X10). The column was washed with water (120 ml.) and elution then begun with 0·5 N-acetic acid, 2 ml. fractions being collected. Fractions 115–125 contained α-aminoadipic acid mixed with relatively small amounts of glutamic acid and an unidentified compound, probably a peptide, which migrated almost as far as glutamic acid on paper electrophoresis in pyridine–acetate buffer (0·05 M with respect to acetate), pH 4·5. These fractions were combined and evaporated to dryness *in vacuo*. To facilitate the subsequent purification of the α-aminoadipic acid, 6 N-HCl (1 ml.) was added to the residue, the solution heated at 105° for 16 hr. and the hydrolysate evaporated to dryness *in vacuo* over solid KOH. The residue was dissolved in water (3 ml.) and subjected to electrophoresis in pyridine–acetate buffer (0·05 M with respect to acetic acid), pH 5·0, in a Beckman Spinco model CP continuous-flow paper-electrophoresis

cell. The cell was run at constant current (80 mA). The sample was fed to the curtain during 18 hr. and 32 fractions were collected. The point of application of the sample was opposite fraction 1, the fractions being numbered from cathode (1) to anode (32). Evaporation of fractions 5–7 yielded a residue (2·48 mg.) in which α-aminoadipic acid was the only ninhydrin-positive compound revealed when a sample was subjected to paper electrophoresis and chromatography.

The remainder of the residue was dissolved in 0·1 N-HCl (1 ml.) and the total amount of α-aminoadipic acid in the solution (2·08 mg.) was estimated by the photometric ninhydrin method (Moore & Stein, 1948). A sample of this solution was diluted threefold and samples (35 μl.) of the diluted solution were used for counting at infinite thinness.

Lysine from mycelium. A sample (1 g.) of the washed freeze-dried mycelium was heated under reflux for 16 hr. in 6 N-HCl (50 ml.). The mixture was filtered and the filtrate evaporated to dryness. The residue was dissolved in water (3 ml.) and the solution applied to a column (2·5 cm. diam. × 13 cm.) of Dowex 1 (200–400 mesh; acetate form; X10). The column was washed with water (300 ml.), and the eluate, containing neutral and basic amino acids, was evaporated to dryness. The residue was dissolved in water (2 ml.) and applied to a column of Dowex 50 (100–200 mesh, pyridine form; X4). Neutral amino acids were eluted with water (100 ml.), and arginine, histidine and lysine, which remained on the column, were then separated by the carrier-displacement technique of Buchanan (1957). The fractions containing lysine were combined and evaporated to dryness. The residue was dissolved in 0·5 N-HCl (1 ml.) and the solution boiled with Norit and filtered. The filtrate was evaporated to dryness, the residue dissolved in boiling 95% (v/v) ethanol (2 ml.), and a mixture of pyridine–ethanol–water (10:10:1, by vol.) added until the pH rose to approximately 4 (bromocresol green). A crystalline precipitate of lysine hydrochloride (19·2 mg.) separated from the mixture, and was recrystallized from ethanol–water (9:1, v/v).

RESULTS

Incorporation of [1-^{14}C]*valine*

The addition of DL-[1-^{14}C]valine to the culture medium resulted in the incorporation of ^{14}C into cephalosporin C (Expt. 1, Table 1). The apparent dilution of molar radioactivity would have been less than half that shown in Table 1 if it had been calculated on the basis of the specific radioactivity of the total free valine in the medium after the [1-^{14}C]valine had been added. About 2·6 mg. of free valine was found to be present initially in 200 ml. of medium B1. The amount of free valine decreased steadily during the fermentation and was no longer detectable after 20 hr. The amount of radioactivity in the respiratory carbon dioxide showed that a substantial proportion of the added valine underwent oxidative degradation. A further substantial proportion was incorporated into the mycelium of the *Cephalosporium* sp.

Radioautographs made after paper electrophoresis and chromatography of an acid hydro-

Table 1. *Incorporation of* ^{14}C*-labelled amino acids into cephalosporin C, respiratory carbon dioxide and mycelium of a* Cephalosporium *sp.*

The procedures used for fermentations in shaken flasks, isolation of cephalosporin C and determination of respiratory $^{14}CO_2$ were as described by Trown *et al.* (1962). Cultures were harvested 70 hr. after inoculation. Valine, the sodium salt of α-aminoadipic acid and the hydrochlorides of the isomers of cystine were added immediately after inoculation as aqueous solutions which had been sterilized by autoclaving. At the end of the fermentations, the mycelium was separated by centrifuging, washed with water, freeze-dried and assayed directly for ^{14}C. Each experiment was with 200 ml. of medium. The yields of cephalosporin C were determined by bioassay. The values for the specific radioactivities of cephalosporin C refer to the product before dilution with unlabelled cephalosporin C.

Expt. no.	Medium	Amino acid added	Yield of cephalosporin C (mg.)	Recovery of added ^{14}C (%)			Specific radioactivity of amino acid added (mC/m-mole) (*A*)	Specific radioactivity of cephalosporin C (μC/m-mole) (*B*)	Apparent dilution of molar radioactivity (*A*/*B*)
				In cephalosporin C	In CO_2	In mycelium			
1	B1	DL-[1-^{14}C]Valine (2·5 mg.; 0·1 mC)	16	1·4	34·1	22·2	4·76	42	113
2	B3	DL-[3-^{14}C]Cystine + *meso*-[3-^{14}C]cystine (approx. 1:1; 13·1 mg.; 0·1 mC)	36	3·1	4·0	31·2	1·83	42	43·5 (74)*
3	B3	DL-α-Amino[2-^{14}C]adipic acid (10·3 mg.; 0·0328 mC)	11	0·38	0·085	6·8	0·51	5·2	98

* Calculated from the molar radioactivity of the glycine derived from C-6 and C-7.

lysate of the [^{14}C]cephalosporin C revealed that a substantial amount of radioactivity was present only in spots corresponding to compounds 1 and 2, but that a trace of radioactivity was present in α-aminoadipic acid. Compounds 1 and 2 are derived from the sulphur and C-2, C-3, C-4, C-9 and C-10 of cephalosporin C (Abraham & Newton, 1961). Paper electrophoresis and chromatography of an acid hydrolysate of the products of hydrogenolysis of cephalosporin C revealed the presence in the hydrolysate of cephalosporidine [formed in about 30 % yield from the α-aminoadipoyl side chain, C-6, C-7 and the nitrogen atom attached to C-7 (Abraham & Trown, 1963)], valine (from C-2, C-3, C-4, C-9 and C-10), glycine (from C-7 and C-8, and/or C-7 and C-6), alanine (from C-6, C-7 and C-8) and α-aminoadipic acid. Radioautographs revealed radioactivity only in the spots corresponding to valine, α-aminoadipic acid and cephalosporidine respectively. Quantitative experiments indicated that about 90 % of the total radioactivity of the cephalosporin C was present in one or more of the carbon atoms incorporated into compounds 1 and 2, i.e. the valine-yielding fragment of the molecule (Table 2).

Incorporation of [3-^{14}C]*cystine*

The ^{14}C in a mixture of DL-[3-^{14}C]cystine and *meso*-[3-^{14}C]cystine was incorporated into cephalosporin C with an apparent dilution of molar radioactivity which was of the same order as that for DL-[1-^{14}C]valine (Expt. 2, Table 1). A larger proportion of the added ^{14}C appeared in the mycelium, and a smaller proportion in the respiratory carbon dioxide, than was the case with [1-^{14}C]valine.

Radioautographs made after paper electrophoresis and chromatography of an acid hydrolysate of the products of hydrogenolysis of the cephalosporin C with Raney nickel showed radioactivity in spots corresponding with cephalosporidine, alanine, glycine, α-aminoadipic acid and valine. The spot due to cephalosporidine was the most radioactive and that due to valine the least. Quantitative experiments (Table 2) showed that the major part of the total radioactivity of the cephalosporin C was present in the fragment of the β-lactam ring (C-6 and C-7) which was obtained in the form of a glycine residue when an acid hydrolysate of cephalosporin C was oxidized with silver oxide, but that significant fractions of the total radioactivity were also present in the α-aminoadipoyl group and the carbon atoms incorporated into compounds 1 and 2.

Incorporation of α-amino[2-^{14}C]*adipic acid*

The ^{14}C of DL-α-amino[2-^{14}C]adipic acid was incorporated into cephalosporin C with an apparent dilution of molar radioactivity which was of the same order as that found in the experiments with labelled valine and cystine (Expt. 3, Table 1). However, very little of the ^{14}C in the added α-aminoadipic acid appeared in the respiratory carbon dioxide and only a relatively small proportion appeared in the mycelium. Radioautographs made after paper electrophoresis and chromatography of an acid hydrolysate of the cephalosporin C revealed radioactivity only in the spot corresponding to α-aminoadipic acid. When hydrolysis was preceded by hydrogenolysis with Raney nickel, radioactivity was found only in spots corresponding to α-aminoadipic acid and cephalosporidine respectively, the total radioactivity in the latter being about half that in the former. Quantitative experiments showed that at least 99 % of the total radioactivity of the cephalosporin C was present in its α-aminoadipic acid residue.

The α-amino[2-^{14}C]adipic acid added to the fermentation had been autoclaved in aqueous solution at pH 7·0 and contained 1·37 % of 6-oxopiperidine-2-carboxylic acid. It was formally possible for the added 6-oxo[2-^{14}C]piperidine-2-carboxylic acid to have been the sole source of the ^{14}C in the cephalosporin C, since the latter contained only 0·38 % of the total radioactivity added to the fermentation (Table 1). However, in a

Table 2. *Distribution of radioactivity in cephalosporin C formed in the presence of* ^{14}C*-labelled amino acids*

Degradation products were obtained from cephalosporin C as described in the text. Compounds 1 and 2 (Abraham & Newton, 1961) were derived from C-2, C-3, C-4, C-9 and C-10; glycine was derived from C-6 and C-7, and CO_2 from C-8.

	Molar radioactivity (as % of that determined for cephalosporin C)				
Amino acid added	α-Amino-adipic acid	Glycine	Compounds 1* and 2	Acetic acid	CO_2
DL-[1-^{14}C]Valine	2	—	90 × 2	2 ± 1	—
DL-[3-^{14}C]Cystine + *meso*-[3-^{14}C]cystine	13	59	11·5 × 2	2·5 × 1	2·5
DL-α-Amino[2-^{14}C]adipic acid	99	—	0·1 × 2	1 ± 0·5	—

* Since compounds 1 and 2 are C_{10} compounds their molar radioactivity is twice that of the C_5 valine-yielding fragment of the cephalosporin C molecule.

similar experiment, a solution of α-amino[2-^{14}C]-adipic acid was autoclaved without neutralization, before addition to the fermentation, under conditions which resulted in the conversion of α-aminoadipic acid into 6-oxopiperidine-2-carboxylic acid to the extent of about 60%. The results of this experiment did not differ significantly from those of Expt. 3 (Table 1).

Other radioactive products from the fermentations

During the isolation of cephalosporin C, which was carried out as described by Trown *et al.* (1962), partially purified material was eluted from a column of Amberlite XE-58 resin with ammonium acetate buffer, and the elution followed by measurements of the extinction at 260 mμ and of radioactivity. The elution curves were qualitatively similar to those obtained during the isolation of cephalosporin C from fermentations to which [1-^{14}C]acetate had been added (Trown *et al.* 1962), in each case consisting of three main bands (*A*, *B* and *C*). Cephalosporin C was located in band *C*.

When [1-^{14}C]valine had been added to the fermentation, the radioactivity curve closely followed the extinction curve. But when [3-^{14}C]cystine and α-amino[2-^{14}C]adipic acid had been added, the peak of radioactivity in band *C* slightly preceded the peak of the extinction. In previous experiments with [1-^{14}C]acetate, a similar displacement of the first peak from the second appeared to be caused partly by the presence of radioactive 6-oxopiperidine-2-carboxylic acid, which is eluted from the column just before cephalosporin C (Trown *et al.* 1962). In the present experiments with labelled cystine and α-aminoadipic acid (Expts. 2 and 3, Table 1), radioactive 6-oxopiperidine-2-carboxylic acid was shown by paper chromatography to be present in the effluent when cephalosporin C was adsorbed on a column of Dowex 50 (X8) and its radioactivity was estimated by direct counting on the paper. In the experiment with [3-^{14}C]cystine, the 6-oxopiperidine-2-carboxylic acid contained 14% of the radioactivity of the effluent, and, in the experiment with α-amino[2-^{14}C]adipic acid, 85%. The latter value corresponded to about 30% of the radioactivity which had been added to the fermentation as 6-oxopiperidine-2-carboxylic acid. Paper chromatography and electrophoresis showed that both effluents contained at least five uncharacterized radioactive compounds which were ninhydrin-negative but could be detected by the chlorination procedure of Rydon & Smith (1952).

With the experiments with [3-^{14}C]cystine and α-amino[2-^{14}C]adipic acid further studies were made of the material in bands *A* and *B* respectively. When the material in band *A* from the fermentation in which [3-^{14}C]cystine had been used was added to a column of Dowex 50 (H^+ form; X4) the effluent contained a radioactive ninhydrin-positive substance which behaved like taurine on paper chromatography and electrophoresis. Material eluted from the column at pH 8 was hydrolysed with 6N-hydrochloric acid at 105° for 16 hr. Radioactive alanine, valine, α-aminoadipic acid and glycine, together with very small amounts of radioactive cystine and cysteic acid (the radioactivity of which accounted for less than 1% of that of the radioactive cystine added) were detected, by paper chromatography and electrophoresis, in the hydrolysate. A large proportion of the radioactivity in the hydrolysate from band *B* appeared in a compound which was ninhydrin-negative, had no net charge at pH 4·5 and had the same R_F as cephalosporidine in butan-1-ol–acetic acid–water. A number of amino acids with no detectable radioactivity were also present in the hydrolysates of the materials from bands *A* and *B*.

When α-amino[2-^{14}C]adipic acid was added to the fermentation, the amount of radioactivity in band *B* was 110 times as large as that in band *A* and 37 times as large as that in band *C*, in contrast with the results obtained with labelled valine and cystine, when all three bands contained similar amounts of radioactivity. The major part of the radioactivity in band *B* (which accounted for 55% of the radioactivity added to the fermentation) was found in free α-aminoadipic acid, but a small amount appeared in a compound which behaved like cephalosporidine on paper electrophoresis in pyridine–acetate buffer, pH 4·5, and paper chromatography in butan-1-ol–acetic acid–water. The specific radioactivity of α-aminoadipic acid isolated from material in band *B* (0·293 mc/m-mole) was 58% of that of the labelled α-aminoadipic acid added to the fermentation. No significant amount of radioactivity was detected in amino acids other than α-aminoadipic acid after paper chromatography and electrophoresis of acid hydrolysates of material from band *A* and from band *B*.

Lysine isolated from an acid hydrolysate of a sample of mycelium from the fermentation to which α-amino[2-^{14}C]adipic acid had been added showed a molar radioactivity of 1·12 μc/m-mole. The radioactivity of this lysine accounted for 20% of the total radioactivity of the mycelium from which it was obtained. The calculated total radioactivity of lysine from the whole of the mycelium corresponded to 1·4% of the radioactivity added to the fermentation as α-aminoadipic acid.

DISCUSSION

The finding that radioactive cephalosporin C produced in the presence of DL-[1-^{14}C]valine contained 90% of its ^{14}C in the C_5 fragment constituted by C-2, C-3, C-4, C-9 and C-10 (I) gives support to

the suggestion that the carbon skeleton of valine is incorporated intact into this fragment. If this is so, the essential difference between the biosynthesis of the dihydrothiazine ring of cephalosporin C and that of the thiazolidine ring of the penicillins may lie in an oxidation, during the former process, in which the *gem*-dimethyl groups of a valine residue become involved.

A very small proportion of the ^{14}C of [1-^{14}C]-valine which was incorporated into cephalosporin was found in the acetoxy and α-aminoadipoyl groupings in the molecule. Some labelling of both these groups would be expected if [^{14}C]carbon dioxide formed from valine during the fermentation could be incorporated into acetate (Trown *et al.* 1962). Some hypothetical reactions by which this might occur if [^{14}C]carbon dioxide reacted with pyruvate to form labelled oxaloacetate have been mentioned by Trown *et al.* (1963).

The finding that labelled cephalosporin C formed in the presence of a mixture of DL-[3-^{14}C]-cystine and *meso*-[3-^{14}C]cystine contained the major fraction of its ^{14}C in a fragment (from C-6 and C-7) obtained as glycine is consistent with the view that cysteine is incorporated intact into the β-lactam ring of cephalosporin C, as it is into that of benzylpenicillin. However, a considerable fraction of the total ^{14}C in the cephalosporin C was also distributed in other parts of the molecule. The presence of ^{14}C in compounds 1 and 2 (from C-2, C-3, C-4, C-9 and C-10) could be accounted for if cystine were degraded to pyruvate, since a currently accepted pathway for the biosynthesis of valine starts from pyruvate and from acetaldehyde–thiamine pyrophosphate, which may itself be formed from pyruvate (Strassman, Thomas & Weinhouse, 1955; Wixom, Shatton & Strassman, 1960). The ^{14}C of [3-^{14}C]pyruvate might thus be expected to be incorporated into C-2 and C-10 of cephalosporin C. R. L. Wixom (personal communication) has shown that some cysteine-desulphydrase activity is present in extracts obtained by ultrasonic disintegration of the mycelium of the *Cephalosporium* sp. Demain & Newkirk (1962) have suggested that the ability of methionine to stimulate the production of cephalosporin C is due to its repression of the formation of cystathionase, an enzyme which appears to deaminate L-cysteine (Rowbury & Woods, 1961). Towards the end of the accepted pathway to valine αβ-dihydroxyisovaleric acid is converted into α-oxoisovaleric acid by a dihydroxy acid dehydratase. This dehydratase, which is widespread in micro-organisms (Wixom, Wikman & Howell, 1961), has been found in the *Cephalosporium* sp. used in the present experiments (Wixom, 1962).

Oxidative decarboxylation of [3-^{14}C]pyruvate to [2-^{14}C]acetate could account for the incorporation of some ^{14}C into the acetoxy and α-aminoadipoyl groups of cephalosporin C. α-Aminoadipic acid should be labelled in C-2, C-3, C-4 and C-5 if it were formed from [2-^{14}C]acetate and α-oxoglutarate into which ^{14}C from acetate was incorporated by the operation of the citric acid cycle (Strassman & Weinhouse, 1953; Trown *et al.* 1962). The finding that the molar radioactivity of the α-aminoadipic acid from cephalosporin C was higher than that of the acetic acid would thus be understandable.

The ^{14}C of cephalosporin C formed in the presence of DL-α-amino[2-^{14}C]adipic acid was present almost exclusively in the α-aminoadipoyl group. Thus α-aminoadipic acid itself is apparently able to act as a precursor of the cephalosporin C side chain, although it remains to be determined whether only one optical isomer of the exogenous amino acid is utilized and whether only cephalosporin C with a D-residue in its side chain can be formed. The isolation of radioactive lysine from the mycelium of the *Cephalosporium* sp. indicates that one biosynthetic pathway to lysine in this organism involves α-aminoadipic acid as an intermediate. However, since only a small proportion of the ^{14}C from the labelled α-aminoadipic acid was incorporated into the mycelium and only a minute fraction entered the respiratory carbon dioxide, it appears that exogenous α-aminoadipic acid played a very restricted metabolic role during the fermentation. A substantial part of the α-amino[2-^{14}C]adipic acid appeared to remain unchanged in the culture fluid, although the amount that was added to the fermentation was less than 3 moles/mole of cephalosporin C formed. But since the molar radioactivity of the α-aminoadipic acid isolated from the culture fluid at the end of the fermentation was 58% of that added immediately after inoculation, some non-labelled α-aminoadipic acid may have been synthesized by the *Cephalosporium* sp. and excreted into the medium. On the basis of the figures given by Windsor (1951) for the amount of α-aminoadipic acid in corn-steep liquor, and on the assumption that none of the other constituents of the medium contained a significant quantity of this amino acid, the dilution of molar radioactivity by unlabelled α-aminoadipic acid in the original culture medium would be less than 10%.

Although the ^{14}C of added α-amino[2-^{14}C]adipic acid was incorporated specifically into the side chain of cephalosporin C, and although α-amino-[2-^{14}C]adipic acid was present in the culture medium throughout the period in which cephalosporin C was being formed, the incorporation was accompanied by nearly a 100-fold dilution of molar radioactivity. Since α-aminoadipic acid can be synthesized by the *Cephalosporium* sp. from acetate, and since very little ^{14}C from the radioactive α-aminoadipic acid added to the culture

medium was found in the mycelium at the end of the fermentation, it may be that both utilization of unlabelled endogenous α-aminoadipic acid (or a precursor of the latter) and a relatively inefficient utilization of extracellular α-aminoadipic acid made appreciable contributions to the dilution of molar radioactivity.

With the possible exception of cystine, none of the small amounts of the ^{14}C-labelled amino acids added to the fermentations stimulated the production of cephalosporin C. The yield of cephalosporin C from the fermentation to which α-aminoadipic acid was added was in fact lower than the average yield from fermentations to which this amino acid was not added. Somerson, Demain & Nunheimer (1961) showed that lysine inhibited the production of benzylpenicillin by *Penicillium chrysogenum* in a complex medium and that this inhibition was overcome by a similar concentration of DL-α-aminoadipic acid, but that higher concentrations of α-aminoadipic acid were themselves inhibitory. However, the concentrations of the amino acids which they used were considerably higher than those employed in the present experiments.

SUMMARY

1. Cephalosporin C which had been produced by a *Cephalosporium* sp. in a complex medium containing DL-[1-^{14}C]valine was radioactive. Its ^{14}C was localized almost exclusively in the C_5 fragment of the dihydrothiazine ring in the molecule which yields valine on hydrogenolysis.

2. The addition of a mixture of DL-[3-^{14}C]-cystine and *meso*-[3-^{14}C]cystine to the culture medium resulted in the production of radioactive cephalosporin C. The major portion of the ^{14}C in the molecule was present in a C_2 fragment of the β-lactam ring. But significant portions were also present in the C_5 fragment of the dihydrothiazine ring and the α-aminoadipoyl side chain respectively.

3. DL-α-Amino[2-^{14}C]adipic acid was synthesized from γ-bromobutyronitrile and diethyl acetamido-[2-^{14}C]malonate. Cephalosporin C which had been produced in a medium to which this amino acid had been added was radioactive and its ^{14}C was present almost exclusively in the α-aminoadipoyl group of the molecule.

4. Lysine isolated from the mycelium of the *Cephalosporium* sp. grown in the presence of DL-α-amino[2-^{14}C]adipic acid was radioactive. But the proportion of the added radioactivity which was recovered in the mycelium was much smaller than was the case with the experiments involving labelled valine and cystine, and less than 0·1 % was recovered in the respiratory carbon dioxide.

5. Possible implications of some of these findings are discussed.

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REFERENCES

Abraham, E. P. & Newton, G. G. F. (1954). *Biochem. J.* **58**, 266.
Abraham, E. P. & Newton, G. G. F. (1961). *Biochem. J.* **79**, 377.
Abraham, E. P. & Trown, P. W. (1963). *Biochem. J.* **86**, 271.
Arnstein, H. R. V. (1957). *Rep. Progr. Chem.* **54**, 339.
Buchanan, D. L. (1957). *J. biol. Chem.* **229**, 211.
Calvin, M., Heidelberger, C., Reid, J. C., Tolbert, R. M. & Yankwich, P. F. (1949). *Isotopic Carbon: Techniques in its Measurement and Chemical Manipulation*, p. 317. New York: John Wiley and Sons Inc.
Demain, A. L. & Newkirk, J. F. (1962). *Bact. Proc.* A, 48.
Gross, D. (1958). *Nature, Lond.*, **181**, 264.
Hirs, C. H. W. (1956). *J. biol. Chem.* **219**, 611.
Jeffery, J. d'A., Abraham, E. P. & Newton, G. G. F. (1960). *Biochem. J.* **75**, 216.
Moore, S. & Stein, W. H. (1948). *J. biol. Chem.* **176**, 367.
Rowbury, R. J. & Woods, D. D. (1961). *Biochem. J.* **79**, 36P.
Rydon, H. N. & Smith, P. W. G. (1952). *Nature, Lond.*, **169**, 922.
Shapira, J., Shapira, R. & Dittmer, K. (1953). *J. Amer. chem. Soc.* **75**, 3655.
Somerson, N. L., Demain, A. L. & Nunheimer, T. D. (1961). *Arch. Biochem. Biophys.* **93**, 238.
Strassman, M., Thomas, A. J. & Weinhouse, S. (1955). *J. Amer. chem. Soc.* **77**, 1261.
Strassman, M. & Weinhouse, S. (1953). *J. Amer. chem. Soc.* **75**, 1680.
Swallow, D. L. & Abraham, E. P. (1958). *Biochem. J.* **70**, 364.
Trown, P. W., Abraham, E. P., Newton, G. G. F., Hale, C. W. & Miller, G. A. (1962). *Biochem. J.* **84**, 157.
Trown, P. W., Sharp, M. & Abraham, E. P. (1963). *Biochem. J.* **86**, 280.
Windsor, E. (1951). *J. biol. Chem.* **192**, 595.
Wixom, R. L. (1962). *Biochem. J.* **84**, 46P.
Wixom, R. L., Shatton, J. B. & Strassman, M. (1960). *J. biol. Chem.* **235**, 128.
Wixom, R. L., Wikman, J. H. & Howell, G. B. (1961). *J. biol. Chem.* **236**, 3257.