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α -Oxoglutarate as a Precursor of the D- α -Aminoadipic Acid Residue in Cephalosporin C

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When cephalosporin C was produced by a *Cephalosporium* sp. in the presence of [1- 14 C]-acetate, 14 C was incorporated mainly into the acetoxy group and the D- δ -(α -aminoadipoyl) group of the molecule. The distribution of 14 C was consistent with the view that the carbon skeleton of the residue of D- α -aminoadipic acid was formed from acetate and the succinate moiety of α -oxoglutarate (Trown, Abraham, Newton, Hale & Miller, 1962). This paper describes experiments on the incorporation of 14 C from α -oxo[5- 14 C]glutarate into cephalosporin C during production on a small scale in a complex culture medium.

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.). Electrophoresis on Whatman no. 1 paper was carried out in pyridine-acetate buffer (0.05M with respect to acetate), pH 4.5, or in $(\text{NH}_4)_2\text{CO}_3$ 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 at pH 8.9 by use of a bromophenol blue reagent as described by Gross (1958, 1961). 6-Oxopiperidine-2-carboxylic acid and cephalosporidine (Abraham & Trown, 1963) were detected by the chlorination method of Rydon & Smith (1952) as described by Trown *et al.* (1962).

Fermentation methods

Maintenance of the culture (*Cephalosporium* sp. C.M.I. 49 137, mutant 8650), preparation of the inoculum and fermentations in shaken flasks (each flask containing 100 ml.

of a complex medium, B3) were carried out as described by Trown *et al.* (1962). Medium B3 was identical with the medium B2 used by Trown *et al.* (1962), except that it contained 4 times the concentration of ammonium acetate and sufficient corn-steep liquor (3.4 g./l.) to give 100 mg. of N/l.

Isolation of 14 C-labelled cephalosporin C

Crystalline cephalosporin C (sodium salt) from two shaken flasks was isolated as described by Trown *et al.* (1962). The product was diluted 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 extinctions at 260 m μ , at which cephalosporin C shows E_{max} .

Radioactive compounds and radioactivity measurements

α -Oxo[5- 14 C]glutarate (3.05 mc/m-mole) was obtained from The Radiochemical Centre, Amersham, Bucks. Radioactivity measurements were made as described by Trown *et al.* (1962). Mycelium, and BaCO_3 from respiratory CO_2 (Trown *et al.* 1962), were counted at infinite thickness. Other samples were counted at infinite thinness.

Degradation of 14 C-labelled cephalosporin C

Estimation of radioactivity in the acetoxy group. Cephalosporin C (sodium salt) (4.1 mg.) in 1.25N-HCl (80 μ l.) was heated at 100° in a sealed Pyrex tube for 1 hr. Any condensate in the upper part of the tube was transferred to the main bulk of the liquid by heating the upper part and cooling the lower part of the tube. After its contents had been frozen in solid CO_2 -ethanol, the tube was opened and immediately placed inside a 20 ml. Quickfit tube. The latter was connected to a high-vacuum pump through a small trap cooled in solid CO_2 -ethanol and the volatile contents of the inner Pyrex tube sublimed into the trap.

The melted sublimate, which consisted mainly of a mixture of aqueous HCl and the acetic acid derived from

the acetoxy group of the cephalosporin C, was diluted with 0.1 ml. of water and the resulting solution stirred with freshly precipitated Ag_2O (50 mg.) at 30° for 1 hr. to remove Cl^- ions. The precipitate was filtered off and washed with water (1 ml.). Washings and filtrate were combined and evaporated to dryness in an air stream at room temperature and the residue was dissolved in water (56.6 μl). A sample (28.3 μl) of this solution was evaporated on a planchet and the radioactivity of the residue measured. A second sample (4.6 μl) of the solution was subjected to electrophoresis on paper at pH 8.9. A spot corresponding to acetate was located with the bromophenol blue reagent and an approximate estimate of the amount of acetate in the sample was made by a comparison of the area of the spot with the areas of spots obtained from known amounts of acetate (Gross, 1958). This enabled an estimate to be made of the molar radioactivity of the acetic acid, since no other radioactive compound was detected on electrophoresis of the sample from cephalosporin C.

Determination of radioactivity in the α -aminoadipic acid residue. The residue remaining after the removal of the acetic acid from the hydrolysate of cephalosporin C was dissolved in water (56 μl) and the solution applied to a column (0.5 cm. diam. $\times$ 2.5 cm.) of Dowex 1 (100–200 mesh; acetate form; X10) previously washed with water. Neutral and basic substances were eluted with water (4 ml.) and elution was then continued with 0.5N-acetic acid, 1 ml. fractions of eluate being collected. α -Amino-adipic acid appeared in fractions 1–3, which were combined and evaporated to dryness. The resulting white crystalline solid was dissolved in 0.1N-HCl (200 μl) and the amount of α -aminoadipic acid in the solution (0.736 mg.) was determined by analysis of a sample (28.3 μl) by the photometric ninhydrin method (Moore & Stein, 1948). A second sample (28.3 μl) of the solution was then evaporated on a planchet and the radioactivity of the residue measured.

A further sample (0.568 mg.) of the α -aminoadipic acid from radioactive cephalosporin C was mixed with 1.60 mg. of unlabelled DL- α -aminoadipic acid and the mixture degraded by the Schmidt reaction, as described by Trown *et al.* (1962), to ornithine (1.34 mg.).

Determination of radioactivity in the C_6 fragment. An estimate was made of the radioactivity in the C_6 , valine-yielding, fragment of cephalosporin C (C-2, C-3, C-4, C-9 and C-10) by a method similar to that described by Trown *et al.* (1962). The combined amounts of compounds 1 and 2, obtained from the C_6 fragment on hydrolysis of cephalosporin C in 1.25N-HCl (Abraham & Newton, 1961), were determined spectrophotometrically.

A solution of cephalosporin C (sodium salt) (2.62 mg.) in 1.25N-HCl (100 μl) was heated in a sealed tube at 100° for 1 hr. The contents of the tube were evaporated thoroughly to dryness (to remove the acetic acid formed on hydrolysis) and the residue was redissolved in 1.25N-HCl (100 μl). The solution was extracted three times with 0.5 ml. of ethyl acetate, the extracts being combined, dried (over anhydrous Na_2SO_4) and evaporated to dryness. The crystalline residue, which consisted of a mixture of compounds 1 and 2, was dissolved in 0.1N-HCl (150 μl) and a sample (35 μl) evaporated on a planchet. Another sample (10 μl) was diluted with 0.1N-HCl (2.99 ml.) and its extinction measured at 240 $\mu\mu$. Compounds 1 and 2 have very nearly the same extinction coefficient at 240 $\mu\mu$ in 0.1N-HCl

($E_{1\text{cm}}^{1\%}$, 580). These compounds were not separated from each other and for the calculation of molar radioactivities were assumed to be present in the mixture in equimolar amounts.

Hydrogenolysis with Raney nickel followed by acid hydrolysis. This was carried out on a sample (4.2 mg.) of cephalosporin C as described by Trown *et al.* (1962).

Isolation and hydrolysis of other fermentation products

Glutamic acid and aspartic acid. During the purification of cephalosporin C by chromatography on a column of Amberlite XE-58 (Trown *et al.* 1962), material was eluted from the column by ammonium acetate buffer in three main bands (A, B and C). Cephalosporin C was present in the last band (C). The eluate corresponding to band A was passed through a column (2.5 cm. diam. $\times$ 10 cm.) of Dowex 50 (H^+ form; X4) and the column washed with 2 bed-volumes of water. Ninhydrin-positive material was then eluted with 4N- NH_3 and the eluate freeze-dried to yield 166 mg. of residue. A sample (50 mg.) of this residue was hydrolysed in 6N-HCl (1 ml.) at 105° for 16 hr. and the hydrolysate evaporated to dryness. The residue was chromatographed in 0.5N-acetic acid on a column (1 cm. diam. $\times$ 30 cm.) of Dowex 1 (100–200 mesh; acetate form; X10). Neutral and basic substances passed rapidly through the column and were followed by α -aminoadipic acid, glutamic acid and aspartic acid respectively, as described by Trown *et al.* (1962). The α -aminoadipic acid, which was present in very small amount, was not isolated. Glutamic acid (1.02 mg.) and aspartic acid (1.37 mg.) were isolated in crystalline form.

Penicillaminic acid (β -sulphovaline). One of the components of band B from the column of Amberlite XE-58 used for the purification of cephalosporin C was probably the penillic acid from penicillin N (Trown *et al.* 1962). The material from band B was freed from ammonium acetate by adsorption on Dowex 50 (X4) as described for the material in band A. The eluate from the resin was freeze-dried to yield 60 mg. of product. A sample (18 mg.) of this product was treated with 1 ml. of performic acid under the conditions described by Hirs (1956). The oxidized product was subjected to electrophoresis in 10% (v/v) acetic acid in a Beckman Spinco model CP continuous-flow paper-electrophoresis cell. The cell was run at constant voltage (800 v). The sample [in 3 ml. of 10% (v/v) acetic acid] was fed to the curtain during 18 hr. and 32 fractions were collected, the fractions being numbered from cathode (1) to anode (32). The point of application of the sample was opposite fraction 16.

Evaporation of fractions 26 and 27 yielded a crystalline ninhydrin-positive compound (1.2 mg.) which was indistinguishable from penicillaminic acid when chromatographed on paper in butan-1-ol-acetic acid-water (4:1:4, by vol.) and subjected to electrophoresis on paper at pH 4.5.

6-Oxopiperidine-2-carboxylic acid. During the process of purification, cephalosporin C was adsorbed on a column of Dowex 50 (H^+ form) as described by Trown *et al.* (1962). Samples of the effluent from this column (42, each 6.4 μl .) were applied side-by-side to a sheet of Whatman no. 1 paper and chromatographed in butan-1-ol-acetic acid-water (4:1:4, by vol.). Markers of 6-oxopiperidine-2-

carboxylic acid were applied at each end and in the middle of the series of samples and the position of these markers after chromatography was revealed by the chlorination procedure of Rydon & Smith (1952). With the positions of the markers as a guide, strips 3 cm. wide, which should have contained any material in the samples with the same R_F (0.65) as 6-oxopiperidine-2-carboxylic acid, were cut from the paper. Material was eluted from the strips with water and the combined eluates yielded a colourless crystalline residue (0.5 mg.) on evaporation to dryness *in vacuo*. The residue was hydrolysed with 2N-HCl (0.5 ml.) at 105° for 16 hr. and the hydrolysate evaporated to dryness. Paper electrophoresis at pH 4.5 revealed the presence in the hydrolysate of α -amino adipic acid and a small amount of ninhydrin-positive material that showed no net charge. A solution of the hydrolysate in water (0.5 ml.) was therefore added to a column (0.5 cm. diam. $\times$ 7 cm.) of Dowex 1 (200–400 mesh; acetate form; X10). Elution was begun with water and, when no further ninhydrin-positive material appeared in the eluate, was continued with 0.5N-acetic acid. α -Amino adipic acid appeared in the first 3 ml. of eluate, which was evaporated to dryness *in vacuo*. The residue was dissolved in water (75 μ l.) and the total amount of α -amino adipic acid in the solution (277 μ g.) was determined by the photometric ninhydrin method (Moore & Stein, 1948). Samples (35 μ l.) of this solution were used for counting at infinite thinness.

RESULTS

Incorporation of ^{14}C into cephalosporin C

The addition of α -oxo[5- ^{14}C]glutarate to the fermentation 30 hr. after inoculation resulted in the incorporation of ^{14}C into cephalosporin C (Table 1). The incorporation was accompanied by an approximately 1000-fold dilution in molar radioactivity. During the isolation process, the distribution of radioactivity in the fractions eluted from a column of Amberlite XE-58 was similar to

that reported by Trown *et al.* (1962) with [^{14}C]-acetate.

Acid hydrolysis of the radioactive cephalosporin C showed that more than 80 % of its radioactivity was located in the δ -(α -amino adipoyl) side chain, though relatively small amounts of radioactivity were present in the acetoxy group and in the C_5 fragment which gives rise to compounds 1 and 2 (Abraham & Newton, 1961). When the α -amino-[^{14}C]adipic acid obtained from cephalosporin C was degraded by the Schmidt reaction (cf. Trown *et al.* 1962) it yielded ornithine whose molar radioactivity was only 1.6 % of that of the α -amino adipic acid. Thus, more than 98 % of the radioactivity of this α -amino adipic acid was located in C-6.

The material obtained by hydrogenolysis of cephalosporin C and subsequent acid hydrolysis was analysed by electrophoresis on paper at pH 4.5 followed by paper chromatography in butan-1-ol-acetic acid-water (4:1:4, by vol.). Spots corresponding to α -amino adipic acid and cephalosporidine (Abraham & Trown, 1963) were radioactive and when counted directly on the paper the former spot showed about twice as much radioactivity as the latter. No radioactivity was detected in spots corresponding to alanine, glycine and valine respectively (cf. Trown, Smith & Abraham, 1963).

Incorporation of ^{14}C into other metabolic products

Glutamic acid and aspartic acid, which were isolated from an acid hydrolysate of the material obtained in band A when cephalosporin C was purified by chromatography on Amberlite XE-58, were radioactive. The molar radioactivities of the glutamic acid (0.344 $\mu\text{C}/\text{m-mole}$) and of the aspartic acid (0.132 $\mu\text{C}/\text{m-mole}$) were about 13 and 5 %

Table 1. *Incorporation of ^{14}C from α -oxo[5- ^{14}C]glutarate into cephalosporin C, mycelium and respiratory carbon dioxide by a Cephalosporium sp.*

The fermentation was with 200 ml. of medium B3 in shaken flasks (for details see text). Thirty hr. after inoculation 0.1 mc (6.23 mg.) of α -oxo[5- ^{14}C]glutarate (3.05 mc/m-mole) was added. The culture was harvested after 70 hr. when it contained 46 mg. of cephalosporin C according to bioassay. Specific radioactivities refer to cephalosporin C undiluted with non-labelled antibiotic.

Fermentation product	Cephalosporin C degradation product	Distribution of total radioactivity (as % of added ^{14}C)	Specific radioactivity ($\mu\text{C}/\text{m-mole}$)	Molar radioactivity (as % of that of cephalosporin C)
Cephalosporin C	—	0.28	2.92	100
—	D- α -Amino adipic acid	—	2.58	84
—	Acetic acid	—	0.088	3
—	Compounds 1 and 2	—	0.029	0.5 $\times$ 2*
Mycelium	—	0.089	—	—
Respiratory CO_2	—	45†	—	—

* Compounds 1 and 2 are C_{10} compounds containing the carbon atoms of the C_5 fragments from two molecules of cephalosporin C (Abraham & Newton, 1961). The molar radioactivity of compounds 1 and 2 is therefore twice that of the C_5 fragment of the cephalosporin C molecule.

† This is a minimum value, since the respiratory CO_2 was not recovered quantitatively under the conditions used (Trown *et al.* 1962).

respectively of the molar radioactivity of the α -aminoadipic acid isolated from cephalosporin C.

Penicillaminic acid, a product of the oxidation of material eluted in band B from the column of Amberlite XE-58, showed a molar radioactivity ($0.015 \mu\text{C}/\text{m-mole}$) which was approximately half that of compounds 1 and 2 from cephalosporin C.

6-Oxopiperidine-2-carboxylic acid, which was obtained in the effluent when cephalosporin C was adsorbed on a column of Dowex 50 during the purification process (cf. Trown *et al.* 1962), gave radioactive spots when samples of the effluent were subjected to chromatography on paper. Acid hydrolysis of the 6-oxopiperidine-2-carboxylic acid yielded α -aminoadipic acid, whose specific radioactivity ($0.94 \mu\text{C}/\text{m-mole}$) was 36 % of that of the α -aminoadipic acid obtained on hydrolysis of the cephalosporin C.

DISCUSSION

When cephalosporin C was formed by the *Cephalosporium* sp. in a medium containing [$1\text{-}^{14}\text{C}$]acetate, ^{14}C was located mainly in C-1 and C-6 of the α -aminoadipic acid residue (Trown *et al.* 1962). This distribution of ^{14}C could be explained by the hypothesis that α -oxo[$5\text{-}^{14}\text{C}$]glutarate was formed from [$1\text{-}^{14}\text{C}$]acetate in the citric acid cycle, and that α -oxoadipate labelled in C-1 and C-6 was formed from [$1\text{-}^{14}\text{C}$]acetate and the succinate moiety of α -oxo[$5\text{-}^{14}\text{C}$]glutarate. The present finding that the α -aminoadipic acid residue of cephalosporin C formed in the presence of α -oxo[$5\text{-}^{14}\text{C}$]glutarate contained almost all its ^{14}C in C-6 is consistent with this hypothesis. The dilution of radioactivity which accompanied the incorporation of ^{14}C into the α -aminoadipic acid residue was similar to that observed in comparable experiments with [$1\text{-}^{14}\text{C}$]acetate (Trown *et al.* 1962). The presence of ^{14}C in cephalosporidine was to be expected, since this degradation product of cephalosporin C is derived from the α -aminoadipoyl side chain and a fragment of the β -lactam ring (Abraham & Trown, 1963).

A small proportion of the ^{14}C in the labelled cephalosporin C was found in compounds 1 and 2, which are derived from a C_5 fragment of the dihydrothiazine ring that yields valine on hydrogenolysis (Abraham & Newton, 1961). The labelling of compounds 1 and 2 conceivably arose through the conversion of α -oxo[$5\text{-}^{14}\text{C}$]glutarate into [^{14}C]oxaloacetate, in the citric acid cycle, and thence to [$1\text{-}^{14}\text{C}$]pyruvate. The latter could be a precursor of [$1\text{-}^{14}\text{C}$]valine (cf. Strassman, Thomas & Weinhouse, 1955) and thus of the valine-yielding fragment of cephalosporin C (Trown *et al.* 1963). Formation of [$1\text{-}^{14}\text{C}$]pyruvate would also account for the isolation of penicillaminic acid with a radioactivity similar to that of the C_5 fragment of cephalosporin C from

material that was probably derived from penicillin N produced during the fermentation (cf. Trown *et al.* 1962). Valine is known to be a precursor of the carbon skeleton of the penicillamine residue in benzylpenicillin (Arnstein & Grant, 1954), and penicillamine yields penicillaminic acid on oxidation.

A further small proportion of the ^{14}C in labelled cephalosporin C was found in the acetoxy group. Hypothetical reactions which would enable [$1\text{-}^{14}\text{C}$]acetate to be formed from α -oxo[$5\text{-}^{14}\text{C}$]glutarate include the fission of oxaloacetate to acetate and oxalate, as suggested by Lewis & Weinhouse (1951), the fission of malate to acetate and glyoxylate [involving the reversal of one of the stages of the glyoxylic acid cycle (Kornberg & Krebs, 1957)], and the formation of labelled citrate followed by conversion of the latter into labelled acetate by the citrate-cleavage enzyme (Srere, 1959, 1961). Hayaishi, Shimazono, Katagiri & Saito (1956) reported that oxaloacetate was converted into oxalate and acetate by an extract of *Aspergillus niger*.

The value for the molar radioactivity of the 6-oxopiperidine-2-carboxylic acid isolated from one of the fractions obtained during the purification process, which was 36 % of that of the α -aminoadipic acid from cephalosporin C, suggested that free α -aminoadipic acid or 6-oxopiperidine-2-carboxylic acid might have been produced by the *Cephalosporium* sp. and excreted into the culture fluid before labelled α -oxoglutarate was added to the fermentation. But the possibility was not excluded that the 6-oxopiperidine-2-carboxylic acid was formed by the breakdown of a more complex metabolic product.

The fact that glutamic acid isolated from another fraction showed a considerably lower molar radioactivity than either the α -aminoadipic acid from cephalosporin C or the 6-oxopiperidine-2-carboxylic acid might be accounted for by the presence of much more glutamic acid than α -aminoadipic acid in the original culture medium, by the biosynthesis of a higher proportion of the glutamic acid before the addition of labelled α -oxoglutarate, or by both. Dilution of radioactivity in intermediates between α -oxoglutarate and oxaloacetate in the citric acid cycle might also have contributed to the relatively low molar radioactivity of the aspartic acid.

SUMMARY

1. Cephalosporin C produced in a complex medium to which α -oxo[$5\text{-}^{14}\text{C}$]glutarate was added contained most of its radioactivity in the δ -(α -aminoadipoyl) side chain of the molecule.

2. Radioactive α -aminoadipic acid obtained from the cephalosporin C was degraded by the

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