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# Incorporation of $^3H$ from δ-(L-α-Amino[4,5-$^3H$]adipyl)-L-cysteinyl-D-[4,4-$^3H$]valine into Isopenicillin N

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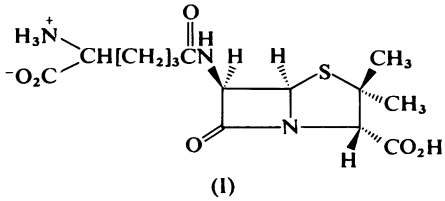
1. δ-(L-α-Amino[4,5-$^3H$]adipyl)-L-cysteinyl-D-[4,4-$^3H$]valine has been synthesized from its constituent amino acids, the L-α-amino[4,5-$^3H$]adipic acid being obtained by reduction with $^3H_2$ of methyl 5-acetamido-5,5-diethoxycarbonylpent-2-enoate and subsequent decarboxylation and hydrolysis. 2. In a cell-free system prepared by lysis of protoplasts of *Cephalosporium acremonium* $^3H$ was incorporated from the doubly labelled tripeptide into a compound that behaved like penicillin N or isopenicillin N. The relative specific radioactivities of the α-aminoadipyl and penicillamine moieties of the penicillin were the same (within experimental error) as those of the α-aminoadipic acid and valine residues respectively of the tripeptide. 3. The behaviour of the labelled α-aminoadipic acid from the penicillin to the L-amino acid oxidase of *Crotalus adamanteus* venom showed that it was mainly L-α-aminoadipic acid. 4. The results are consistent with the hypothesis that the carbon skeleton of the LLD-tripeptide is incorporated intact into the penicillin molecule and that the first product is isopenicillin N.

It has been shown that $^3H$ from the tripeptide δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine radioactively labelled in its valine residue is incorporated into a substance resembling penicillin N (Newton & Abraham, 1954) in a system prepared by lysis of protoplasts of *Cephalosporium acremonium* (Fawcett *et al.*, 1976). However, in these experiments penicillin N (I) could not be distinguished from isopenicillin N, which has an L-α-aminoadipyl group in place of a D-α-aminoadipyl side chain. The work described in the present paper was designed to establish which penicillin is formed first from the LLD-tripeptide and to confirm a previous conclusion that the tripeptide is incorporated without hydrolysis into the penicillin molecule.

## Experimental

### *Materials*

DL-Aminoadipic acid and catalase (from bovine liver) were from the Sigma Chemical Co., Poole, Dorset, U.K. *Crotalus adamanteus* (Eastern diamondback rattlesnake) venom was supplied by Koch-Light Laboratories, Colnbrook, Bucks., U.K. Pyruvate kinase was from the Boehringer Corp. (London) Ltd., London W.C.2, U.K. Dimethyl sulphoxide was dried by refluxing over $CaH_2$ and then distilled. Methyl 4-bromocrotonate, from Aldrich Chemical Co., Gillingham, Dorset, U.K., was dried over $CaCl_2$ in diethyl ether, and diethyl acetamidomalonate (Aldrich Chemical Co.) was dried over $P_2O_5$ *in vacuo*. NaH was freed from oil by washing with diethyl ether dried over $CaCl_2$. Other chemicals were from BDH Chemicals, Poole, Dorset, U.K., and were of AnalaR grade where possible. Silica gel $HF_{254}$ was from E. Merck, Darmstadt, Germany.



(I)

### *Fluorography*

Dried paper chromatograms (35cm × 43cm) were dipped in toluene (scintillation grade) containing 2,5-diphenyloxazole (20%, w/v) and allowed to dry in air. Kodak X-Omat-H film was pre-exposed, as described by Lasky & Mills (1975), with a Metz Mecablitz III flash unit (Fürth, Bavaria, Germany) at a distance of 90cm. The surface of the film that had been nearest the light source was placed in contact with the dried paper and stored in the dark at −70°C for various periods from 1 to 30 days. After the 30 days spots were visible on the developed film where the radioactivity was 0.5nCi/cm² or more.

*Synthesis of* L-α-*amino*[4,5-$^3H$]*adipic acid*

*Methyl 5-acetamido-5,5-diethoxycarbonylpent-2-enoate.* NaH (82.4mg, 3.4mmol) was dispersed in dimethyl sulphoxide (20ml) and stirred for 10min. Diethyl acetamidomalonate (0.75g, 3.4mmol) was added and stirring was continued. After a further 30min methyl 4-bromocrotonate (0.6g, 3.4mmol) was added and the mixture was left overnight. Dimethyl sulphoxide was removed *in vacuo*, excess NaH was destroyed by treatment with aq. 50% (v/v) ethanol (10ml) and the mixture was concentrated to an oil.

Water was added to the oil and the product was extracted into diethyl ether (2×50ml). The ethereal solution was dried over $MgSO_4$ and the diethyl ether was removed in a rotary evaporator to yield 1.1g of oil. The product crystallized from light petroleum (b.p. 40–60°C) and after drying *in vacuo* had m.p. 65–67°C (yield 42%). A sample was recrystallized for elementary analysis (Found: C, 53.1; H, 6.8; N, 4.5; $C_{14}H_{21}NO_7$ requires: C, 53.3; H, 6.7; N, 4.4%). T.l.c. in ethanol/chloroform (1:19, v/v) on silica gel $HF_{254}$ plates showed a single spot at $R_F$ 0.8. Mass spectrometry gave a molecular ion with *m/e* 315.

DL-α-*Amino*[4,5-$^3H$]*adipic acid.* Methyl 5-acetamido-5,5-diethoxycarbonylpent-2-enoate (1mmol) in ethyl acetate was reduced with $^3H_2$ (25Ci) over a Pd/C catalyst at The Radiochemical Centre, Amersham, Bucks., U.K. Hydrogenation of the double bond was completed with $^1H_2$. After removal of the catalyst and labile $^3H$ the product was refluxed for 2h in 4M-HCl to bring about hydrolysis and decarboxylation and the solution was evaporated to dryness *in vacuo*. The crude DL-α-aminoadipic acid had incorporated 20Ci of $^3H$. It was diluted with unlabelled DL-α-aminoadipic acid to give material with a specific radioactivity of 1Ci/mmol.

A portion (5Ci) of the product from The Radiochemical Centre was mixed with unlabelled DL-α-aminoadipic acid (7.25g) in water (30ml) and the solid brought into solution by addition of 6M-HCl. The pH of the solution was then raised to 3.2 with 10M-NaOH. After storage at 4°C for 3 days the DL-α-amino[4,5-$^3H$]adipic acid was removed by filtration, washed with water, ethanol and diethyl ether and dried *in vacuo* (yield, 7.65g). After electrophoresis at pH6.5 it gave a single ninhydrin-positive spot coincident with a single peak of radioactivity. The $^3H$ n.m.r. spectrum of the $^3H$-labelled DL-α-aminoadipic acid was determined by Professor J. A. Elvidge and Mr. J. A. Bloxsidge, Department of Chemistry, University of Surrey, Guildford, Surrey, U.K., the corresponding $^1H$ n.m.r. spectrum being used for the assignment of signals (Al-Rawi *et al.*, 1976). The $^1H$ chemical shifts at C-2, C-3, C-4 and C-5 of α-aminoadipic acid (III) were 4.50, 2.27, 2.10 and 2.67 respectively and the intensities (%) of the corresponding $^3H$ signals were 0, 0, 71 and 29.

The radioactive DL-amino acid (approx. 1g) was further diluted and the product resolved by the method of Greenstein *et al.* (1953). The resulting L-α-amino[4,5-$^3H$]adipic acid (1.1g) had m.p. 208–210°C, $[\alpha]_D^{20}$+25 (*c* 1.98 in 6M-HCl). Its specific radioactivity was 67mCi/mmol. The corresponding D-α-aminoadipic acid had $[\alpha]_D^{20}$−25 (*c* 1.98 in 6M-HCl).

D-[4,4-$^3H$]*Valine*

This was prepared as described by Fawcett *et al.* (1976). The specific radioactivity of the sample used was 67mCi/mmol.

δ-(L-α-*Amino*[4,5-$^3H$]*adipyl*-L-*cysteinyl*-D-[4,4-$^3H$]-*valine*

This peptide was synthesized from labelled L-α-aminoadipic acid (sp. radioactivity 67mCi/mmol) and labelled D-valine (sp. radioactivity 67mCi/mmol) by the procedure used for the synthesis of the corresponding peptide labelled only in its valine residue (Fawcett *et al.*, 1976). The synthesis was carried out on an approx. 0.05mM scale. The final product (33μCi) was analysed by fluorography after paper chromatography in butan-1-ol/acetic acid/water (4:1:4, by vol.; upper phase) and by electrophoresis on paper at pH1.8, 4.5 and 6.5 (Fawcett *et al.*, 1976). The analyses showed major spots corresponding to the tripeptide and its S–S dimer respectively and two further spots due to uncharacterized impurities. Both of these impurities gave radioactive α-aminoadipic acid and valine on acid hydrolysis and both were converted by oxidation with performic acid into a compound that behaved like δ-(α-aminoadipyl)-cysteicylvaline on electrophoresis at pH1.8. Before use of the radioactive peptide an excess of dithiothreitol was added to its aqueous solution to convert the S–S dimer into the free thiol form. The latter was estimated to be about 40% pure by paper chromatography and fluorography, the film being scanned by a microdensitometer (Joyce, Loebl and Co., Gateshead, Tyne and Wear, U.K.).

*Preparation and use of a cell-free system from C. acremonium*

A cell-free enzyme system from protoplasts of *C. acremonium* C91 was prepared as described by Fawcett *et al.* (1976), except that 4-morpholinepropanesulphonic acid (pH7.0) was used as the buffer.

Incubation of the enzyme system with the labelled tripeptide (approx. 1.9μCi; 14nmol) in a total volume of 0.5ml was carried out as described by Fawcett *et al.* (1976), except that the experiment was done with two 0.5ml samples and the control consisted of a

portion (0.5ml) of the enzyme system that had been held in a boiling-water bath for 10min and then cooled in ice before addition of the peptide.

*Analysis of the products of reaction in the cell-free system*

(*a*) After incubation at 27°C for 2h the mixtures were centrifuged at 15000*g* for 10min and the supernatants were removed and freeze-dried. Amino acids, peptides and β-lactam antibiotics were extracted into cold 70% (v/v) ethanol (2×0.5ml). Ethanol was removed by evaporation *in vacuo* and the solutions from the two original 0.5ml samples were combined and freeze-dried. The resulting product was diluted with 80μg of penicillin N purified as described by Smith *et al.* (1967).

(*b*) A sample (15%) of the freeze-dried extract was analysed by fluorography after electrophoresis at pH6.5 (70V/cm for 35min) and chromatography (17h at 4°C) in the second dimension in propan-1-ol/pyridine/acetic acid/acetonitrile/water (40:30:9:40:36, by vol.) (Whitney *et al.*, 1972) before and after treatment of the extract with purified β-lactamase I from *Bacillus cereus* 569/H/9 (Davies *et al.*, 1974) at a final concentration of 12.5μg/ml for 30min at 20°C, to convert the labelled penicillin into its penicilloate.

(*c*) The remainder of the penicillinase-treated extract was subjected to electrophoresis on paper at pH6.5 (70V/cm for 35min). The radioactive penicilloate (located by fluorography) was then eluted from the paper with water. Penicillin N penicilloate migrated 28.2cm towards the anode under these conditions, while the tripeptide and one of its radioactive impurities migrated 20.5cm and a second impurity 12.7cm.

(*d*) The freeze-dried penicilloate was hydrolysed *in vacuo* in 6M-HCl at 105°C for 18h and then oxidized with performic acid (1.5%) as described by Smith *et al.* (1967).

(*e*) A sample (one-fifth) of the material obtained after hydrolysis and oxidation was examined by electrophoresis on paper at pH4.5 (70V/cm for 45min). The radioactivity in the positions corresponding to α-aminoadipic acid and penicillaminic acid was determined as described by Fawcett *et al.* (1976). The counting efficiency was 16%. Determinations of the approximate amounts (to±20%) of α-aminoadipic acid and penicillaminic acid present were made after treatment of the paper with $Cd^{2+}$–ninhydrin (Heilmann *et al.*, 1957) by visual comparison of the spots from the experimental sample with those from a series of dilutions of unlabelled authentic α-aminoadipic acid and penicillaminic acid.

(*f*) The labelled α-aminoadipic acid was eluted from paper after electrophoresis at pH4.5 (70V/cm for 45min) and its optical configuration was determined by use of the L-amino acid oxidase in *Crotalus adamanteus* venom. A solution of the dried venom (10mg/ml) in 0.2M-Tris/HCl buffer, pH7.2, was dialysed in Visking tubing at 4°C for 2.5h. To 1ml of the non-diffusible enzyme solution (after adjustment of the pH to 7.8 with Tris) was added 0.1M-KCl (0.6ml), 25mg of catalase/ml (10μl), 50mM-DL-α-aminoadipic acid (0.2ml) and the labelled α-aminoadipic acid (0.2ml; 30–90nCi) from the penicilloate. Authentic samples of L-α- and D-α-[4,5-$^3$H]aminoadipic acid were mixed similarly with enzyme. Further samples were used for controls lacking enzyme. Each solution was incubated at 27°C for 3.5h in a 10ml Erlenmeyer flask on a rotary shaker (200rev./min). The reaction was stopped by the addition of acetic acid (0.1ml) and each solution applied to a column (2cm×0.5cm) of Dowex 50 (X4; $H^+$ form). The column was washed with water (5ml) and the retained unoxidized α-aminoadipic acid was then eluted with 1M-$NH_3$ (3ml). The radioactivity present in 100μl of the water eluate and in 60μl of the 1M-$NH_3$ eluate was measured. The counting efficiencies were 23 and 24% respectively.

Control experiments showed that the oxidation of L-α-amino[4,5-$^3$H]adipic acid by the L-amino acid oxidase was complete within 2.5h under the conditions used.

*Estimation of endogenous δ-(α-aminoadipyl)cysteinylvaline and penicillin N in the cell-free system*

(*a*) A system prepared by lysis of protoplasts of *C. acremonium* (1ml) was centrifuged at 15000*g* for 10min. The supernatant was freeze-dried and the residue extracted twice with 0.4ml of 70% (v/v) ethanol. The combined extracts were freeze-dried and the residue was oxidized with performic acid and analysed by two-dimensional electrophoresis and chromatography on paper as described by Loder & Abraham (1971). After treatment of the paper with $Cd^{2+}$–ninhydrin reagent, the intensity of the spot due to the sulphonic acid form of the tripeptide was compared visually with the intensities of spots given by known amounts of authentic δ-(α-aminoadipyl)-cysteicylvaline under similar conditions. The approximate value obtained for the amount of endogenous tripeptide in the extract was assumed to apply to other preparations of the cell-free system after adjustments had been made for the quantity of protoplasts used by determination of their DNA content as described by Fawcett *et al.* (1973).

(*b*) The differential activities of isopenicillin N and penicillin N against *Staphylococcus aureus* and *Salmonella typhi* (Usher *et al.*, 1975) enabled it to be shown that the endogenous penicillin was largely penicillin N. Subsequently the amount of endogenous penicillin N in the 15000*g* supernatant from a cell-free system from protoplasts was determined by bioassay as described by Smith *et al.* (1967), but with

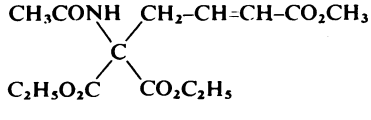
*Escherichia coli* Ess 22–31 (supersensitive to β-lactam antibiotics), which was kindly supplied by Professor A. L. Demain (Massachusetts Institute of Technology, Cambridge, MA., U.S.A.), as the test organism.

## Results

### *Synthesis of δ-(L-α-amino[4,5-$^3H$]adipyl-L-cysteinyl-D-[4,4-$^3H$]valine*

The method of synthesis of the tripeptide used in these experiments from its amino acids was similar to that described by Fawcett *et al.* (1976) for the synthesis of the peptide labelled only in the methyl groups of its D-valine residue. To obtain a doubly labelled tripeptide a relatively simple procedure was devised for the production of radioactive L-α-aminoadipic acid in quantity. This depended on the synthesis of methyl 5-acetamido-5,5-diethoxycarbonylpent-2-enoate (II) from diethyl acetamidomalonate and methyl 4-bromocrotonate. Reduction of the double bond with $^3H_2$ in the presence of a Pd/C catalyst followed by acid hydrolysis and decarboxylation of the product yielded $^3H$-labelled DL-α-aminoadipic acid with a specific radioactivity of about 20 Ci/mmol, which was suitably diluted with unlabelled DL-α-aminoadipic acid (III) before resolution into its optical isomers and further use. The $^3H$ n.m.r. spectrum of the labelled DL-α-aminoadipic acid showed that $^3H$ was attached only to C-4 and C-5 (III), but that the ratio of $^3H$ at C-4/$^3H$ at C-5 was 2.45:1. An analogous asymmetric distribution has been found after hydrogenation of other αβ-unsaturated carbonyl compounds. Its occurrence is related to the nature of the catalyst used and to the *cis* or *trans* isomerism at the double bond. One hypothesis is that a 1:4 addition occurs followed by migration to the β-position of a proton originating from either the carboxy group or the gas phase (Simon & Berngruber, 1968). In the present case the completion of the reduction with $H_2$ might be accompanied by exchange of $^3H$ for $^1H$ at C-5 of the α-aminoadipic acid.

Analysis by paper chromatography and electrophoresis of the small amount of tripeptide finally obtained from labelled valine and α-aminoadipic acid indicated that it was about 40% pure. However, the product contained no free α-aminoadipic acid or valine and its almost total conversion into the sulphonic acid form of the tripeptide of oxidation with performic acid indicated that the impurities were closely related to δ-(L-α-amino[4,5-$^3$H]adipyl)-L-cysteinyl-D-[4,4-$^3$H]valine. The product was therefore used in biosynthetic experiments without further attempts at purification.



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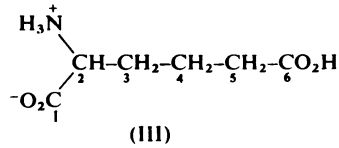

(III)

### *Incorporation of $^3H$ from the doubly labelled tripeptide into isopenicillin N*

To determine the incorporation of $^3$H from δ-(L-α-amino[4,5-$^3$H]adipyl)-L-cysteinyl-D-[4,4-$^3$H]valine into the α-aminoadipyl side chain and the penicillamine (D-β-thiovaline) moiety respectively of the penicillin formed in the cell-free system, the penicillin was first diluted with unlabelled penicillin N and converted into its penicilloate by treatment with β-lactamase I from *B. cereus*. The penicillin was not resolved satisfactorily from an impurity in the labelled tripeptide by two-dimensional electrophoresis and chromatography, but the labelled penicilloate was readily separated from other labelled compounds by electrophoresis on paper at pH 6.5. After hydrolysis with 6M-HCl and oxidation with performic acid the penicilloate yielded α-aminoadipic acid and penicillaminic acid. The distribution of radioactivity after electrophoresis of a sample of the mixture (14% of the total from 1 ml of enzyme system) on paper at pH 4.5 is shown in Fig. 1. The radioactivities of the segments containing α-aminoadipic acid and penicillaminic acid were 58 and 39 nCi respectively and the corresponding amounts of these amino acids were 15.5±3.1 and 12.7±2.5 nmol (mean±S.D.). The specific radioactivities of the α-aminoadipic acid and penicillaminic acid were thus 3.8±0.8 and 3.1±0.6 μCi/μmol (mean±S.D.) respectively.

The specific radioactivity of the $^3$H-labelled tripeptide used in these experiments was 134 μCi/μmol and 14 nmol was added to each 0.5 ml of the enzyme system. Since this amount of enzyme system contained about 3 nmol of unlabelled tripeptide the specific radioactivity of the peptide from which incorporation of $^3$H into the penicillin occurred was 110 μCi/μmol. On the assumption that this was also the specific radioactivity of the penicillin formed, the measured radioactivity of its α-aminoadipic acid residue and penicillamine moiety corresponded to that from 7.5 and 5.1 nmol respectively of penicillin per ml of enzyme system. The corresponding estimated yields of penicillin from the tripeptide in the system were thus 22 and 15%. The higher value could well be lower than the true one, since the calculations suppose that α-aminoadipic acid and penicillaminic acid were isolated from the penicillin in 100% yield.

*Optical configuration of the penicillin α-aminoadipyl side chain*

Experiments with authentic $^3$H-labelled L- and D-α-aminoadipic acid showed that the L- and D-isomers were clearly distinguished by treatment of the amino acid with L-α-amino acid oxidase and separation on Dowex 50 of the remaining amino acid from the oxo acid formed. Nevertheless, 9 and 2% of the radioactivity of the L- and D-α-aminoadipic acids respectively failed to be retained on the column of Dowex 50 before treatment with the enzyme and 6% of the radioactivity of the L-isomer was retained after treatment (Table 1). From the results of experiments with the α-amino[4,5-$^3$H]adipic acid from the penicillin produced in the presence of the doubly labelled tripeptide it was evident that this amino acid was mainly the L-isomer and therefore that the penicillin was mainly isopenicillin N (Table 1). If it were assumed that the small retention of the authentic L-isomer was due to incomplete oxidation, and that there was complete retention of $^3$H from the unoxidized D-isomer from penicillin, then the proportion of the D-isomer in the sample would be 11%. However, what proportion of the $^3$H retained on the Dowex 50 was due to impurities is uncertain. Hence, no precise value can be given for the small proportion of penicillin N in the penicillin formed.

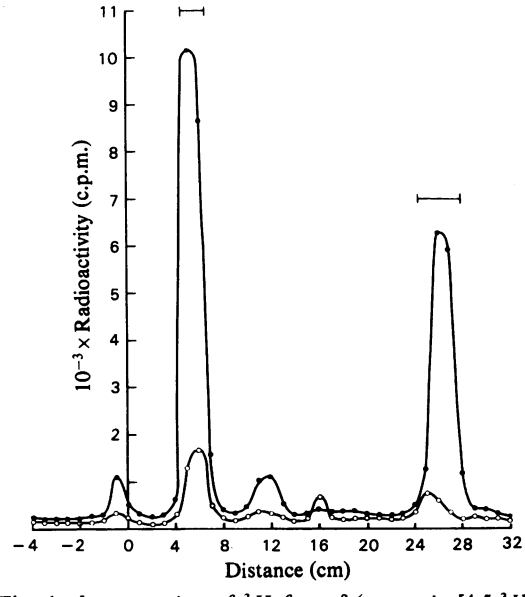


Fig. 1. *Incorporation of $^3$H from δ-(L-α-amino[4,5-$^3$H]-adipyl)-L-cysteinyl-D-[4,4-$^3$H]valine into the α-aminoadipyl and penicillamine moieties of a penicillin penicilloate*
Electrophoresis on paper of the products of hydrolysis and oxidation of the penicilloate was at pH 4.5 for 45min with 14% of the total sample from 1ml of enzyme system. The values for radioactivity (c.p.m.) were from segments of paper (each 1cm × 3cm). The bars show the position of α-aminoadipic acid (left) and penicillaminic acid (right) as revealed by $Cd^{2+}$–ninhydrin. Symbols: ●, profile of radioactivity after an experiment with the active enzyme system; ○, profile after a control experiment with the boiled enzyme system.

## Discussion

The specific radioactivities of the α-aminoadipyl and penicillamine moieties of the penicillin formed in the cell-free system from *C. acremonium* in the presence of δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (equally labelled with $^3$H in its α-aminoadipic acid and valine residues) were the same within the experimental error. After dilution of radioactivity by the addition of unlabelled penicillin N before analysis of the reaction product, the measured specific radioactivities of the isolated α-aminoadipic acid and penicillaminic acid were about 3.5nCi/nmol. Corresponding values of 1.9nCi/nmol can be calculated from the amount of unlabelled penicillin N added (202nmol), the amount of unlabelled endogenous penicillin N present in 1ml of enzyme system (approx.

Table 1. *Behaviour of different samples of α-aminoadipic acid to L-amino acid oxidase*
Samples of radioactive α-aminoadipic acid were incubated with L-amino acid oxidase from *Crotalus adamanteus* (for details see the Experimental section). The reaction mixture was applied to a column of Dowex 50 (X4; $H^+$ form) and the radioactivity in the water and 1M-$NH_3$ eluates was determined. The values in line (*a*) represent the mean for two determinations after incubation of the samples with enzyme. Individual values differed by ±0.7nCi to ±2.4nCi from the mean. The values in line (*b*) represent control determinations from samples without enzyme.

| Sample | | Radioactivity (nCi) from Dowex 50 in: Water eluate | 1M-$NH_3$ eluate | Radioactivity (% of total) in: Water eluate | 1M-$NH_3$ eluate |
|---|---|---|---|---|---|
| L-α-Amino[4,5-$^3$H]adipic acid | (*a*) | 31.8 | 2.0 | 94 | 6 |
| | (*b*) | 3.5 | 33.9 | 9 | 91 |
| D-α-Amino[4,5-$^3$H]adipic acid | (*a*) | 5.1 | 63.2 | 7 | 93 |
| | (*b*) | 1.5 | 65.2 | 2 | 98 |
| α-Aminoadipic acid from $^3$H-labelled penicillin | (*a*) | 77.6 | 13.4 | 85 | 15 |
| | (*b*) | 15.5 | 71.4 | 18 | 82 |

5nmol) and the radioactivity in the isolated α-aminoadipic acid, on the assumption that the specific radioactivity of the penicillin formed is that of the tripeptide precursor (110nCi/nmol). It seems likely that the labelled tripeptide was incorporated into the penicillin without dilution of radioactivity and that the lower value for the calculated specific radioactivity is due to the isolation of α-aminoadipic acid and penicillaminic acid in low yields.

The results presented here are entirely consistent with incorporation of the LLD-tripeptide into the penicillin with its carbon skeleton intact. If hydrolysis had preceded incorporation, a considerable and uneven dilution of radioactivity would have been expected, since the amount of added tripeptide (14nmol/0.5ml) was much less than the corresponding amounts of valine (140nmol) and α-aminoadipic acid (60nmol) found previously in the system (Fawcett, 1975).

The initial concentration of the doubly labelled tripeptide used in the present paper in the cell-free system was 31 μM, which was 10 to 15 times lower than the concentration of δ-(L-α-aminoadipyl)-L-cysteinyl-D-[4,4-$^3$H]valine used in similar experiments by Fawcett *et al.* (1976). Despite the decrease in the concentration of the peptide, the estimated amount of penicillin formed increased more than 3-fold. However, no firm conclusions about the reasons for the increase can be reached on the basis of the present data, because of the possible variations in the activity of different preparations of the cell-free system.

The finding that the penicillin formed in the cell-free system was mainly isopenicillin N indicates that the latter is the first penicillin synthesized from the LLD-tripeptide and that the synthesis involves no change in the optical configuration of either the α-aminoadipic acid or the valine residue. This is consistent with a previous failure to detect the incorporation of $^3$H into a penicillin from the DLD-tripeptide (Fawcett *et al.*, 1976). Since intact cells of *C. acremonium* excrete penicillin N the D-α-aminoadipyl side chain of the latter presumably arises by epimerization after formation of the penicillin ring system. Whether this is brought about by a membrane-bound enzyme remains to be investigated. Konomi *et al.* (1979) have shown independently by a differential microbiological assay that isopenicillin N is formed on addition of L-α-aminoadipyl-L-cysteinyl-D-valine to a cell-free system similar to the one used in the present paper.

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