Use of α -Aminoadipic Acid for the Biosynthesis of Penicillin N and Cephalosporin C by a *Cephalosporium* sp.

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1. L- α -Amino[6- 14 C]adipic acid has been prepared from the DL-amino acid by oxidation of the L-isomer with L-amino acid oxidase to α -oxo[6- 14 C]adipic acid and by transamination of the latter with L-glutamic acid in an extract of a *Cephalosporium* sp. prepared by ultrasonic treatment of the mycelium. 2. The optical configuration of small amounts of 14 C-labelled α -aminoadipic acid from the mycelium of the *Cephalosporium* sp. has been determined by treatment with L-amino acid oxidase and measurement of the proportion of radioactivity subsequently retained on a column of a strong cation-exchange resin. 3. α -Aminoadipic acid which had been labelled in the mycelium from [1- 14 C]acetate appeared to contain more than 99% of the L-isomer. 4. L- α -Amino[14 C]adipic acid (sodium salt) was taken up much more rapidly than the D-isomer, or α -oxo[6- 14 C]adipic acid, by suspensions of washed mycelium of the *Cephalosporium* sp. in water. The pool of intracellular α -aminoadipic acid was expandable. 5. Intracellular products found to be labelled with 14 C from L- α -amino[14 C]adipic acid were δ -aminovaleric acid, saccharopine, lysine, protein, compounds which behaved like penicillin N, cephalosporin C and deacetylcephalosporin C respectively on paper chromatography and electrophoresis, and a peptide whose amino acid residues include α -aminoadipic acid, cysteine and valine. 6. L- α -Amino[14 C]adipic acid acted as a precursor of the δ -(D- α -aminoadipoyl) side chains of extracellular penicillin N and cephalosporin C. 7. 14 C from D- α -amino[14 C]adipic acid was incorporated into penicillin N and cephalosporin C, but the incorporation was accompanied by a relatively high dilution of specific radioactivity and some L- α -amino[14 C]adipic acid was found in the intracellular pool. 8. These findings are discussed in relation to the origin of the D- configuration of the α -aminoadipoyl side chain of the antibiotics.

Penicillin N and cephalosporin C, produced by a *Cephalosporium* sp., are δ -(D- α -aminoadipoyl) derivatives of 6-aminopenicillanic acid and 7-aminocephalosporanic acid respectively (Abraham & Newton, 1954, 1961). Isopenicillin, which differs from penicillin N only in having a side chain derived from the L-isomer of α -aminoadipic acid, has been obtained from *Penicillium chrysogenum* (Flynn, McCormick, Stamper, De Valeria & Godzeski, 1962; Cole & Batchelor, 1963). The tripeptide δ -(α -aminoadipoyl)cysteiclylvaline was reported to be present in extracts of *P. chrysogenum* that had been treated with performic acid, but the optical configuration of its α -aminoadipic acid residue was not ascertained (Arnstein & Morris, 1960).

These findings with *P. chrysogenum* suggested that L- α -aminoadipic acid is involved in the bio-

synthesis of benzylpenicillin by this organism. Other findings appeared to give support to this view. The production of benzylpenicillin was reported to be inhibited by L-lysine and the inhibition reversed by DL- α -aminoadipic acid (Somerson, Demain & Nunheimer, 1961). Since L- α -aminoadipic acid can act as a precursor of L-lysine, a possible explanation of the inhibitory effect of the latter was that it repressed the biosynthesis of the former.

D- α -Aminoadipic acid appears to have been found hitherto in Nature only in penicillin N and cephalosporin C. The finding that radioactivity from [1- 14 C]acetate was incorporated into C-1 and C-6 of the α -aminoadipic acid residue of cephalosporin C and that radioactivity from α -oxo[5- 14 C]glutarate was incorporated into C-6 indicated that the carbon skeleton of the side chain of this antibiotic was formed from acetylcoenzyme A and the succinate moiety of α -oxoglutarate (Trown,

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Abraham, Newton, Hale & Miller, 1962; Trown, Sharp & Abraham, 1963a). Experiments with DL- α -amino[2- 14 C]adipic acid showed that 14 C from the added amino acid was incorporated into the side chain of cephalosporin C but did not reveal whether the L- or D-isomer, or both, acted as a precursor (Trown, Smith & Abraham, 1963b).

The experiments described in this paper were designed to provide a comparison of the metabolism of L- and D- α -aminoadipic acid by washed mycelium and cell-free extracts of the *Cephalosporium* sp. and to show whether the addition of 14 C-labelled optical isomers of α -aminoadipic acid to the mycelium would throw light on the origin of the D-configuration in the α -aminoadipoyl side chain of the antibiotics.

METHODS

Antibacterial assays of penicillin N and cephalosporin C, paper chromatography and electrophoresis, the location of substances on paper by bioautography, radioautography and coloration with ninhydrin, and the counting of radioactive compounds on paper and on planchets were carried out as described by Smith, Warren, Newton & Abraham (1967). The radioactivities (R_{μ} C) of samples of solutions used for paper chromatography and electrophoresis were determined from counts of the samples and of a polymer reference source at infinite thinness on planchets. If the count/min. on paper of the sample applied was C_1 and the count/min. from a given spot after running was C_x the radioactivity of the spot was taken to be $R.C_x/C_1 \mu$ C.

Preparations of mycelium

Suspensions of washed mycelium. *Cephalosporium* sp. mutant 8650 was grown in a chemically defined medium in shake flasks and the mycelium was harvested 72 hr. after inoculation, washed with water, and resuspended in water as described by Smith *et al.* (1967). Flasks containing suspensions of washed mycelium were shaken (200 rev./min.) at 27.5°. In some experiments samples (2 ml.) of suspensions in water were shaken at 120 oscillations/min. in 20 ml. Warburg vessels. The vessels contained 3 N-KOH in the centre cup and were gassed with O_2 .

Mycelium subjected to ultrasonic treatment. Washed mycelium was suspended in 0.1 M-tris-HCl buffer, pH 7.4, containing 0.1 M-KCl, 0.01 M-MgSO₄ and 0.001 M-L-cysteine hydrochloride. The suspension (5 ml. of buffer/g. of damp-dry mycelium) was subjected to ultrasonic treatment for 30 min. as described by Smith *et al.* (1967). In some cases the treated suspension was then separated into a supernatant fraction and a pellet by centrifugation at 20000g for 30 min. The supernatant fraction contained a thin layer of lipid on the surface of a clear aqueous layer. The latter was used for studies on transamination.

Unlabelled compounds

L- and D- α -Aminoadipic acid. These compounds were kindly prepared by Mrs M. Sharp by treatment of *N*-chloroacetyl-DL- α -aminoadipic acid with hog acylase I (from

British Drug Houses Ltd., Poole, Dorset) as described by Greenstein, Birnbaum & Otey (1953) except that *N*-chloroacetylation of DL- α -aminoadipic acid was carried out with *p*-nitrophenyl chloroacetate.

Chloroacetic acid (2.36 g.) and *p*-nitrophenol (4.18 g.) were dissolved in ethyl acetate (75 ml.). The solution was cooled in ice and stirred and a solution of dicyclohexylcarbodi-imide (5.15 g.) in ethyl acetate was added. A white precipitate formed almost immediately. Stirring was continued for 30 min. with the solution cooled in ice and then for 1.5 hr. at room temperature. Acetic acid (0.25 ml.) was added and the urea derivative removed by filtration. Evaporation of the filtrate yielded yellow crystalline *p*-nitrophenyl chloroacetate, m.p. 95–97° (yield 80%). After crystallization from ethanol containing a little acetic acid the compound had m.p. 98–99°. A sample prepared by Buyle (1964) had m.p. 94°.

DL- α -Aminoadipic acid (8.09 g.) was dissolved in a solution (81 ml.) of 0.965 N-NaOH containing Na₂CO₃ (5.28 g.). *p*-Nitrophenyl chloroacetate (10.67 g.) in acetone (100 ml.) was added to the solution (pH 10.0). After 1 hr. acetone was removed *in vacuo*, the pH of the aqueous residue adjusted to 5.5 with N-HCl and the mixture extracted seven times with 150 ml. of ethyl acetate to remove the precipitated *p*-nitrophenol. The pH of the aqueous phase was then adjusted to 1.5 with 6 N-HCl and the solution extracted seven times with 150 ml. of ethyl acetate. The extracts were combined, dried over Na₂SO₄ and the ethyl acetate was removed *in vacuo*. The residue of crude *N*-chloroacetyl-DL- α -aminoadipic acid weighed 8.6 g. (yield 73%). After recrystallization from ethyl acetate it had m.p. 129–130°. Greenstein *et al.* (1953) give m.p. 129°.

α -Oxoadipic acid. This was prepared by condensation of diethyl glutarate with diethyl oxalate in the presence of sodium ethoxide and hydrolysis of the resulting triethyl 2-carboxy-1-oxoadipate, as described by Gault (1912). The product, recrystallized from CHCl₃, had m.p. 125–127°. A sample prepared by Gault (1912) had m.p. 127°.

α -Oxopimelic acid. This was prepared by the procedure used for the preparation of α -oxoadipic acid, except that diethyl glutarate was replaced by diethyl adipate. After crystallization from CHCl₃ it had m.p. 90–92°. A sample prepared by Adickes (1925) had m.p. 93–94°.

Radioactive compounds

L-[U- 14 C]Lysine (13.4 μ C/ μ mole), [1- 14 C]acetate (35.9 μ C/ μ mole) and diethyl acetamido[2- 14 C]malonate (1.8 μ C/ μ mole) were obtained from The Radiochemical Centre, Amersham, Bucks. DL- α -Amino[6- 14 C]adipic acid (4.4 μ C/ μ mole) was obtained from Calbiochem, Lucerne, Switzerland. DL- α -Amino[2- 14 C]adipic acid was prepared as described by Trown *et al.* (1963b). The D-isomer (0.92 μ C/ μ mole) was prepared from this product by the procedure used for the preparation of D- α -amino[6- 14 C]adipic acid.

D- α -Amino[6- 14 C]adipic acid and α -oxo[6- 14 C]adipic acid. A sample (11.35 μ moles) of DL- α -amino[6- 14 C]adipic acid (4.4 μ C/ μ mole) and unlabelled L- α -aminoadipic acid (5 μ moles) were placed in one side arm of a Warburg vessel in 0.2 ml. of water containing an equivalent of Na₂CO₃. Unlabelled L- α -aminoadipic acid (10 μ moles) and an equivalent of Na₂CO₃ were placed in 0.2 ml. of water in a second side arm. The amino acid in the first side arm was

then allowed to react with L-amino acid oxidase from *Crotalus adamanteus* as described by Smith *et al.* (1967). When O₂ uptake ceased, unlabelled L- α -aminoadipic acid was added from the second side arm and oxidation allowed to continue to completion. When the theoretical amount of O₂ had been consumed the contents of the flask were applied to a column (7 cm. $\times$ 1 cm. diam.) of Dowex 50 (X 4; H⁺ form; 200–400 mesh) and the latter was washed with water (20 ml.). After electrophoresis on paper at pH 4.5 of a small sample of the combined percolate and washing, the radioactivity of the latter was found in a position corresponding to that of α -oxoadipic acid. The main portion of this solution, containing α -oxo[6-¹⁴C]adipic acid, was evaporated to dryness *in vacuo*. D- α -Amino[6-¹⁴C]adipic acid was eluted from the column with 2N-pyridine (30 ml.) and the eluate evaporated to dryness *in vacuo*.

L- α -Amino[6-¹⁴C]adipic acid. A solution of α -oxo[6-¹⁴C]adipic acid (5.5 μ moles; 2.34 μ C/mole) in water (5 ml.) was freed from traces of D- α -amino[6-¹⁴C]adipic acid by passage through a column (10 cm. $\times$ 1 cm. diam.) of Dowex 50 (X 4; H⁺ form; 200–400 mesh). The column was washed with water (35 ml.) and the combined percolate and washings were evaporated to dryness. The residue was dissolved in water (10 ml.) and a sample (30.3 μ l.) of the solution counted. The pH of the main portion of the solution (containing 15 μ C) was adjusted to 7.0 with N-NaOH and the solution freeze-dried. The product was dissolved in water (0.1 ml.) containing sodium L-glutamate (27 μ moles) and the solution transferred to a stoppered tube. A sample (4 ml.) of supernatant from mycelium subjected to ultrasonic treatment was added and the mixture kept at 27.5° for 90 min. Samples (5 μ l.) of the reaction mixture before and after incubation were subjected to electrophoresis at pH 4.5. Before incubation, 0.8% of the ¹⁴C applied to the paper appeared in the position of α -aminoadipic acid; after incubation 70% appeared in this position.

After incubation, the main portion of the solution was applied to a column (10 cm. $\times$ 1 cm. diam.) of Dowex 50 (X 4; H⁺ form), the column washed with water (30 ml.) and elution carried out with N-NH₃ (35 ml.). The radioactivity of the percolate and washings corresponded to 3.17 μ C and that of the eluate to 11.09 μ C.

The eluate was evaporated to dryness, the residue dissolved in water (1.0 ml.) and the solution applied to a column (30 cm. $\times$ 1 cm. diam.) of Dowex 1 (X 10; acetate form; 200–400 mesh). Elution was begun with 0.5N-acetic acid and fractions (2 ml.) were collected every 5 min. Samples (5 μ l.) of each fraction were subjected to electrophoresis on paper at pH 4.5 and the radioactivities of further samples (35 μ l.) determined by counting on planchets. Most of the radioactivity was present in fractions 16, 17 and 18, which contained L- α -amino[6-¹⁴C]adipic acid contaminated with a small proportion of unlabelled glutamic acid. These fractions were pooled and evaporated to dryness. The residue was dissolved in water, the pH of the solution adjusted to 7.0 with 0.1N-NaOH, and the solution freeze-dried. The radioactivity of the L- α -amino[6-¹⁴C]adipic acid (sodium salt) corresponded to 10.45 μ C (yield from α -oxo[6-¹⁴C]adipic acid 69.5%).

Optical configuration of α -aminoadipic acid in mycelial extracts. This was determined by estimation of the proportion of ¹⁴C-labelled α -aminoadipic acid, obtained from mycelial extracts, which behaved as a substrate for L-amino acid oxidase. The method was adapted from that of Katz

& Weissbach (1963). The enzyme was used in Warburg vessels as described by Smith *et al.* (1967). In a typical experiment, the α -aminoadipic acid was labelled by ¹⁴C from [1-¹⁴C]acetate.

Isolation of α -amino[¹⁴C]adipic acid from mycelium. Suspensions (each 25 ml.) of washed mycelium in water were incubated for 30 min. in two 250 ml. shake flasks after [1-¹⁴C]acetate (10 μ C; 0.279 μ mole) had been added to each flask. The mycelium was harvested and treated with cold 70% (v/v) ethanol, followed by cold water, as described by Smith *et al.* (1967), to extract compounds in the intracellular pool. The aqueous solution remaining after ethanol had been removed from the combined extracts *in vacuo* (total radioactivity 8.47 μ C) was applied to a column (15 cm. $\times$ 1 cm. diam.) of Dowex 50 (X 4; H⁺ form; 200–400 mesh). The column was washed with water (60 ml.) and α -amino[¹⁴C]adipic acid and other radioactive amino acids (total radioactivity 3.93 μ C) were eluted with 2N-pyridine (60 ml.).

The eluate was evaporated to dryness and the residue applied in water (1 ml.) to a column (40 cm. $\times$ 1 cm. diam.) of Dowex 1 (X 10; acetate form; 200–400 mesh). The column was washed with water (150 ml.) and elution carried out with 0.5N-acetic acid. Fractions (4 ml.) were collected every 10 min. Analysis of samples (5 μ l.) from each fraction by electrophoresis on paper and coloration of spots with ninhydrin indicated that α -aminoadipic acid and a small proportion of glutamic acid was present in fractions 21–23 and glutamic acid, but no α -aminoadipic acid, in fractions 24–26. Fractions 21–23 (total radioactivity 2.96 μ C) were combined and evaporated to dryness.

To remove virtually all of the radioactive glutamic acid with which this preparation of α -aminoadipic acid was contaminated the latter was mixed with unlabelled L-glutamic acid and unlabelled L- α -aminoadipic acid (each 5 mg.) and the mixture rechromatographed three times on Dowex 1 (X 10; acetate form) under the conditions used with the first column. Fractions 29–31 from the final column, containing largely α -aminoadipic acid (total radioactivity 0.206 μ C) were combined, the pH of the solution adjusted to 7.0 and the solution freeze-dried. Electrophoresis on paper at pH 4.5 of the product obtained from the final column followed by radioautography and counting, indicated that 95.4% of the total radioactivity was present in α -aminoadipic acid and 4.6% in glutamic acid.

Oxidation of α -amino[¹⁴C]adipic acid with L-amino acid oxidase. The preparation of α -amino[¹⁴C]adipic acid (sodium salt) was dissolved in water (0.1 ml.) and unlabelled sodium L- α -aminoadipate (10 μ moles) in water (0.1 ml.) was added. The solution was placed in one side arm of a Warburg vessel and unlabelled sodium L- α -aminoadipate (10 μ moles) in water (0.2 ml.) in the other. A solution of L-amino acid oxidase was prepared and used as described by Smith *et al.* (1967) and the reaction carried out at 38° with air as the gas phase. The contents of the first side arm were mixed with the enzyme and the vessel was shaken until no further uptake of O₂ was observed. The contents of the second side arm were then also mixed with the enzyme. The overall O₂ uptake was about 95% of the estimated theoretical uptake. The product (0.1425 μ C) was applied to a column (10 cm. $\times$ 1 cm. diam.) of Dowex 50 (X 4; H⁺ form; 200–400 mesh), the column washed with water (30 ml.) and adsorbed amino acid eluted with N-NH₃ (35 ml.). Most of the radioactivity (92.9%) appeared in

the percolate and washing, but a significant amount (7.1%) appeared in the eluate. The eluate was therefore evaporated to dryness, the residue mixed with unlabelled sodium L- α -aminoadipate (10 μ moles) and the mixture treated with L-amino acid oxidase as before. The theoretical amount of O_2 was adsorbed. When the product was analysed on a column of Dowex 50 (X 4), as before, except that elution was carried out with 2N-pyridine, 44.8% of the radioactivity appeared in the percolate and 55.2% in the eluate. Hence the maximum proportion of D-isomers in the α -aminoadipic acid was 7.1% of 55%, i.e. 3.92%. Electrophoresis on paper at pH 4.5 of a sample of the eluate, followed by radioautography, revealed no radioactivity in the position corresponding to α -aminoadipic acid.

RESULTS

Uptake of D- and L- α -aminoadipic acid and α -oxoadipic acid by aerated washed mycelium

L- α -Amino[6- ^{14}C]adipic acid. Fig. 1 shows the percentage disappearance of radioactivity with time from the extracellular fluid when solutions of L- α -amino[6- ^{14}C]adipic acid sodium salt (pH 7.1) containing different amounts of amino acid but the same amount of radioactivity (0.0499 μ C/ml.) were added to suspensions in water of washed mycelium in shake flasks at 27°.

Curves 1 and 2 (Fig. 1) indicate that the rate of uptake of this amino acid was approximately proportional to its extracellular concentration until the latter approached 0.67 μ mole/ml., the velocity constant (k) calculated for a first-order process being about 0.013 min.⁻¹. With a further

eightfold increase in concentration, however, the rate of uptake increased less than fivefold. Lineweaver-Burk plots of the results of the two experiments gave V_{max} as 0.83 and 0.39 μ mole/min./ml. of suspension respectively and K_m as 4.0 and 3.1 mM respectively.

After the addition of L- α -amino[^{14}C]adipic acid to mycelial suspensions (initial concentration 0.204 μ mole/ml.) the major portion of the radioactivity of the mycelium during a period extending to 3 hr. was found in free α -aminoadipic acid in the intracellular pool. This radioactivity increased almost linearly for about 1 hr. and showed only a relatively small decrease during the next 2 hr. (Table 1).

Semiquantitative estimations of the amounts of α -aminoadipic acid in the amino acid pool were made by electrophoresis at pH 4.5 of samples of mycelial extracts and visual assessment of the intensities of spots coloured with ninhydrin. These indicated that the α -aminoadipic acid pool was expandable. After mycelium had been suspended for 4 hr. in shake flasks in water containing 1.56 μ moles of α -aminoadipic acid/ml. the internal concentration of the amino acid was about six times that in the mycelium of a control suspension in water alone and about 25 times the extracellular concentration.

D- α -Amino[6- ^{14}C]adipic acid. Under similar conditions the D-isomer of α -amino[6- ^{14}C]adipic acid was taken up much less rapidly than its optical antipode. At concentrations between 0.03 and 0.62 μ mole/ml. the rate of uptake appeared to be approximately proportional to the concentrations, but the calculated values of k were between 0.001 min.⁻¹ and 0.0025 min.⁻¹ with different batches of mycelium. Thus in one experiment less than 30% of the initial amounts of D- α -aminoadipic acid (10 and 100 μ g./ml. respectively) disappeared from the extracellular fluid in 6 hr. A Lineweaver-Burk plot gave V_{max} as 0.0025 μ mole/min./ml. of suspension and K_m as 3.6 mM.

In a further experiment unlabelled D- α -aminoadipic acid (ammonium salt) and D- α -amino[6- ^{14}C]adipic acid (sodium salt) were added to washed mycelium suspended in water in a shake flask to give 0.163 μ C and 5.86 μ moles of D-amino acid/ml. of suspension fluid. The flask was shaken for a further 3.5 hr. By this time, 30.5% of the radioactivity had disappeared from the extracellular fluid. The mycelium was then separated by filtration, washed with water and resuspended in the original volume of water containing no amino acid. When counted immediately after resuspension, the extracellular fluid contained 0.22% of the radioactivity added initially to the first suspension. After the second suspension had been shaken for 3.5 hr. only a further 1.37% of the initial radio-

Fig. 1. Uptake of L- α -amino[^{14}C]adipic acid by mycelial suspensions of *Cephalosporium* sp. 8650 in shake flasks. The curves represent results with different initial concentrations of the labelled amino acid but with the same amount of radioactivity. ●, 0.1007 μ mole/ml.; ○, 0.674 μ mole/ml.; ×, 5.14 μ moles/ml.; Δ, 30.64 μ moles/ml.

Table 1. *Uptake and distribution of ^{14}C from L- α -amino[^{14}C]adipic acid by suspension of washed mycelium*

Three flasks (100ml.) each containing 2g. of damp-dry mycelium (harvested 72hr. after inoculation) and 12ml. of water were shaken for 60min. (200 rev./min.) at 27.5°. L- α -Amino[6- ^{14}C]adipic acid (0.47 μC /ml.: 0.204 μmole /ml.; 2.3 μC /mole) was then added to one flask. A sample (0.2ml.) was withdrawn from this flask immediately for measurement of the initial concentration of α -amino[^{14}C]adipic acid. Subsequently samples (2.0ml.) were withdrawn at intervals from all three flasks. For methods of analysis see the text. The mycelium was harvested 180min. after addition of α -amino[^{14}C]adipic acid.

Time (min.)	^{14}C found (% of total added)								
	Extracellular fluid		Intracellular fluid			Hot-tri-chloroacetic acid extract		Hydrolysate of mycelial residue	
	Total	α -Amino adipic acid	Total	α -Amino-adipic acid	Lysine	Saccharo-pine	Protein		
0	100	100	0	0	0	0	0	0	0
10	97.3	90.7	5.2	5.1	0.06	0.07	0.07	0.59	0.17
30	80.1	79.9	17.8	15.9	0.4	0.26	0.84	0.62	0.17
60	59.8	55.0	32.3	31.7	1.75	0.55	2.43	1.85	0.17
180	34.8	27.2	41.9	35.2	5.42	0.51	4.95	7.68	1.48

activity appeared in the extracellular fluid and part, at least, of this was presumably due to the excretion of labelled antibiotics.

α -Oxo[6- ^{14}C]adipic acid. When α -oxo[6- ^{14}C]adipic acid was added as its sodium salt (pH 7.0) to suspensions of washed mycelium in water radioactivity disappeared from the extracellular fluid more slowly than with D- α -aminoadipic acid under similar conditions. At initial concentrations of 0.657 and 6.5 μmoles /ml. respectively the rate of uptake was about 1%/hr. during 6hr. This suggested that the rate was approximately proportional to the concentration over these ranges of concentrations, with k about 0.00017 min. $^{-1}$.

Features of the metabolism of L- and D- α -aminoadipic acid and α -oxoadipic acid

Optical configuration of α -aminoadipic acid in the amino acid pool. α -Amino[^{14}C]adipic acid was isolated from the intracellular pool of washed mycelium of *Cephalosporium* sp. 8650 which had been shaken in water for 30min. after the addition to the suspension of sodium [1- ^{14}C]acetate (final concn. 0.4 μC /ml.; 0.011 μmole /ml.). After treatment of the product with L-amino acid oxidase only about 4% of its radioactivity was retained on a column of Dowex 50 (H^+ form). However, the α -amino[^{14}C]adipic acid was contaminated with a small proportion of glutamic acid, which accounted for about 4% of its radioactivity. L-Glutamic acid is not susceptible to oxidation by L-amino acid oxidase (Ratner, 1955). The radioactivity in the eluate from the column was largely found in glutamic acid and no α -amino[^{14}C]adipic acid was detected. Hence the α -aminoadipic acid was virtually (probably > 99%) the L-isomer.

Conversion of L- α -aminoadipic acid into lysine in suspensions of washed mycelium. Table 1 shows the change with time of the radioactivity in the extracellular fluid and in fractions of the mycelium after the addition of L- α -amino[6- ^{14}C]adipic acid to suspensions of washed mycelium of the *Cephalosporium* sp. in water. It also shows the distribution of the ^{14}C in the amino acid pool between α -aminoadipic acid, saccharopine and lysine. In the earliest (10min.) sample the labelling of saccharopine was similar to that of lysine. But after 60min. the amount of radioactivity in saccharopine had reached a low value, whereas that in lysine was more than three times as great and continued to increase.

No evidence was obtained that the overall process leading from L- α -aminoadipic acid to lysine was reversible to any significant extent. When L-[U- ^{14}C]lysine (final concn. 1.0 μC /ml.; 0.075 μmole /ml.) was added to suspensions of washed mycelium in shake flasks it was taken up rapidly, less than 5% of the added radioactivity remaining in the extracellular fluid after 35min. Of the ^{14}C in the intracellular pool after 6hr., 61% was present in lysine and none was detected in α -aminoadipic acid. However, a small but significant amount was found in compounds which behaved like glutamic acid and saccharopine respectively when subjected to electrophoresis and chromatography on paper. Acid hydrolysis of the mycelial residue (after removal of the intracellular pool) yielded no radioactive compounds except lysine and traces of glutamic acid.

After addition of L-lysine hydrochloride (final concn. 11 μmoles /ml.) or L- α -aminoadipic acid (0.62 μmole /ml.), or both, to suspensions of washed mycelium in water in shake flasks, the amounts of

penicillin N and cephalosporin C produced in 4 hr. did not differ significantly from those in control flasks containing no added amino acid.

D- α -Aminoadipic acid. Six hours after the addition of D- α -amino[6- 14 C]adipic acid (0.642 μ C/ml.; 0.146 μ mole/ml.) to a shaken suspension of washed mycelium in water, 66% of the radioactivity had disappeared from the extracellular fluid and 97% of this radioactivity was recovered from the intracellular pool. Small amounts of radioactivity were found in the hot-trichloroacetic acid and in the sodium hydroxide (protein) extracts. Electrophoresis on paper at pH 4.0 followed by chromatography in butan-1-ol-acetic acid-water showed that about 2% of the radioactivity in this pool was in lysine, 0.4% in a base that behaved like δ -aminovaleric acid and 90.5% in α -aminoadipic acid. The α -aminoadipic acid was isolated by chromatography on Dowex 1 (X 10; acetate form), mixed with unlabelled L- α -aminoadipic acid and the mixture allowed to react with L-amino acid oxidase. Analysis of the product on Dowex 50 (H⁺ form) indicated that the radioactive α -aminoadipic acid from the pool contained about 77% of the D-isomer. On the assumption that the intracellular pool of 1 g. of damp-dry mycelium occupied 0.6 ml. and that intracellular α -aminoadipic acid was distributed uniformly in the pool the quotient (concn. of intracellular D- α -aminoadipic acid)/(concn. of extracellular D- α -aminoadipic acid) at the end of this experiment was about 10.

α -Oxoadipic acid in suspensions of washed mycelium. Seven hours after the addition of α -oxo[6- 14 C]adipate (2.37 μ C/ μ mole) to a suspension of washed mycelium, about 2% of the added 14 C was found in the intracellular pool. However, no detectable amount of this radioactivity was present in α -oxoadipic acid. Radioautography after paper electrophoresis and chromatography indicated that most of the intracellular 14 C was present in α -aminoadipic acid.

α -Oxoadipic acid in preparations of mycelium broken by ultrasonic treatment. Suspensions of washed mycelium which had been subjected to ultrasonic treatment in tris-hydrochloric acid buffer were separated into a supernatant fraction and a pellet by centrifugation at 20 000g for 20 min. The supernatant contained about 3.7 mg. of protein/ml. α -Oxo[6- 14 C]adipic acid (sodium salt; 0.326 μ C; 0.293 μ mole) was added to a sample of the supernatant (4 ml.) in a 50 ml. shake flask and the mixture shaken at 27° for 2 hr. When the mixture was added to a column (10 cm. $\times$ 1 cm. diam.) of Dowex 50 (H⁺ form) and the latter washed with water (35 ml.), 85% of the radioactivity was retained on the column. The material associated with the radioactivity on the column was eluted with 2N-pyridine (35 ml.) and behaved like α -

aminoadipic acid when subjected to electrophoresis on paper at pH 4.5 and chromatography on paper in butan-1-ol-acetic acid-water. The residue obtained on evaporation of the eluate was mixed with unlabelled sodium L- α -aminoadipate and the optical configuration of the α -amino[14 C]adipic acid in the mixture was estimated from the amount of radioactivity which remained unabsorbed on a column of Dowex 50 (X 4; H⁺ form) after two successive treatments with L-amino acid oxidase. The results indicated that more than 97.5% of the α -aminoadipic acid which had been formed from α -oxoadipic acid was the L-isomer.

Further experiments were done to determine which fraction of the sonic extracts catalysed the formation of L- α -aminoadipic acid from the keto acid and the conditions under which the reaction occurred. A semiquantitative estimate of the amount of α -aminoadipic acid formed was made by electrophoresis at pH 4.5 of appropriate samples from fractions to which α -oxo[14 C]adipate had been added 1 hr. previously.

After the addition of α -oxoadipate to suspensions of intact mycelium no extracellular α -amino[14 C]adipic acid was detected. The amino acid was formed rapidly from α -oxo[14 C]adipate in the presence of the clear supernatant obtained by centrifugation of mycelial suspensions subjected to ultrasonic treatment, but none was detected in the presence of the pellet resuspended in buffer. The supernatant still showed considerable activity after dialysis against buffer at 4° for 24 hr. and there was little, if any, increase in activity on addition of pyridoxal phosphate (1–50 μ g./ml.). The optimum pH for the reaction was between 7 and 9. When the pH was decreased to 6 or 5, the formation of α -aminoadipic acid was greatly decreased but the formation of three new compounds was revealed by electrophoresis at pH 4.5. One migrated to the position of β -alanine and gave a characteristic sky-blue colour with ninhydrin. The second migrated about half as far as lysine towards the cathode and was indistinguishable from γ -aminobutyric acid. The third, which migrated slightly further and was present only in very small amounts, behaved like δ -aminovaleric acid. These compounds were presumably formed from aspartic acid, glutamic acid and α -aminoadipic acid respectively by an amino acid decarboxylase (or decarboxylases) which showed significant activity only below pH 7.0.

No significant increase in the formation of α -aminoadipic acid from the corresponding keto acid was observed when ammonium chloride, D- or L-aspartic acid, D-alanine or D-glutamic acid was added to the supernatant from broken mycelium. However, a three- to four-fold increase resulted from the addition of L-glutamic acid and a two- to three-fold increase from the addition of L-alanine.

Table 2. Incorporation of ^{14}C into extracellular penicillin N and cephalosporin C from α -amino[^{14}C]adipic acid by mycelial suspensions

With L- α -amino[6- ^{14}C]adipic acid (initial concentration in suspension 0.47 $\mu\text{C}/\text{ml.}$; 0.204 $\mu\text{mole}/\text{ml.}$) the conditions were those described in Table 1, except that 1 g. of mycelium in 6 ml. of water was shaken in a 50 ml. flask. With DL- α -amino[6- ^{14}C]adipic acid (1.025 $\mu\text{C}/\text{ml.}$; 0.233 $\mu\text{mole}/\text{ml.}$) experiments were carried out in Warburg vessels (for details see the text). With D- α -amino[2- ^{14}C]adipic acid (0.8 $\mu\text{C}/\text{ml.}$; 0.87 $\mu\text{mole}/\text{ml.}$) and D- α -amino[6- ^{14}C]adipic acid (0.64 $\mu\text{C}/\text{ml.}$; 0.146 $\mu\text{mole}/\text{ml.}$) the conditions were those used with the L-isomer. The amounts of antibiotics appearing in the suspension fluid were determined by biological assay.

Precursors added	Time after addition (min.)	Penicillin N			Cephalosporin C		
		Yield ($\mu\text{mole}/\text{ml.}$)	Dilution of sp. radioactivity	Incorporation of ^{14}C (% of total added)	Yield ($\mu\text{mole}/\text{ml.}$)	Dilution of sp. radioactivity	Incorporation of ^{14}C (% of total added)
L- α -Amino[6- ^{14}C]adipic acid	30	0.012	9.2	0.66	0.009	6.9	0.65
	60	0.023	12.4	0.9	0.017	5.5	1.54
	180	0.055	9.8	2.7	0.041	4.5	4.5
DL- α -Amino[6- ^{14}C]adipic acid	60	0.033	8.6	1.4	0.038	5.2	2.6
	120	0.033	7.5	1.6	0.075	5.6	4.7
	240	0.058	8.6	2.4	0.088	4.7	6.9
D- α -Amino[2- ^{14}C]adipic acid	420	0.276	15.4	2.1	0.073	13.8	0.61
D- α -Amino[6- ^{14}C]adipic acid	360	0.11	25.9	2.8	0.098	24.4	2.7

The α -aminoadipic acid produced from α -oxo-[6- ^{14}C]adipic acid in the presence of L-glutamic acid was isolated by chromatography on Dowex 1 (X 10; acetate form). Manometric experiments with L-amino acid oxidase showed that virtually all the α -aminoadipic acid formed was the L-isomer. A similar experiment showed that in the presence of added D-glutamic acid the α -aminoadipic acid formed was more than 91% L.

Addition of α -oxopimelic acid, in place of α -oxoadipic acid, to the supernatant resulted in the formation of a small amount (corresponding to about 10% of the keto acid added) of a ninhydrin-positive compound that behaved in the manner expected of α -aminopimelic acid when subjected to electrophoresis on paper at pH 4.5.

Incorporation of α -amino[^{14}C]adipic acid into penicillin N, cephalosporin C and related compounds

L- α -Aminoadipic acid. The incorporation of radioactivity from added L- α -amino[6- ^{14}C]adipic acid into penicillin N and cephalosporin C produced by suspensions of washed mycelium in water is shown in Table 2. Between 30 and 180 min. after addition of the labelled amino acid the concentrations of the antibiotics in the extracellular fluid increased more than fourfold, but there were only relatively small changes in their specific radioactivities.

Similar experiments were carried out with DL- α -amino[6- ^{14}C]adipic acid. In one case estimates

were made of the radioactivity in both extracellular and intracellular antibiotics and also in intracellular material ('ACVSO₃H') detected in the intracellular pool after oxidation with performic acid that behaved like the sulphonic acid of δ -(α -aminoadipoyl)cysteinylvaline when subjected to electrophoresis on paper at pH 1.8 (Table 3). Within 30 min. about 40% of the radioactivity of the added DL-amino acid was found in α -aminoadipic acid in the intracellular pool (Table 3). This radioactivity was almost entirely accounted for by uptake of the L-isomer. During the following 210 min. it underwent relatively little change, presumably because the metabolism of the ^{14}C -labelled L-isomer was approximately balanced by the slow uptake of ^{14}C -labelled D-isomer. The radioactivity in the intracellular products which behaved like penicillin N, cephalosporin C and ACVSO₃H respectively was in each case from 1 to 3% of that in the intracellular α -aminoadipic acid, and a similar radioactivity was found after radioautography in a position corresponding to deacetylcephalosporin C. The dilution of specific radioactivity which accompanied the incorporation of ^{14}C into intracellular antibiotics was similar to that found with α -amino[L- ^{14}C]adipic acid.

D- α -Aminoadipic acid. When D- α -amino[6- ^{14}C]adipic acid was added to suspensions of washed mycelium in water ^{14}C was incorporated into penicillin N and cephalosporin C (Table 2). After 6 and 7 hr. the incorporation was accompanied by a dilution of specific radioactivity between two and

Table 3. Incorporation of ^{14}C from DL- α -amino[^{14}C]adipic acid intracellular compounds

DL- α -Amino[6- ^{14}C]adipic acid ($4.4\ \mu\text{C}/\mu\text{mole}$) was added to suspensions of washed mycelium in water in shake flasks to give $1.025\ \mu\text{C}/\text{ml}$. Intracellular compounds were extracted with 70% (v/v) ethanol, subjected to electrophoresis (pH 4.0 or pH 1.8) and chromatography on paper and radioactive spots counted on paper as described in the Methods section. Values in parentheses are those for penicillin N and cephalosporin C in the suspension fluid.

Time from addition of α -amino[^{14}C]- adipic acid (min.)	^{14}C (% of total added)			
	Penicillin N*	Cephalosporin C*	'ACVSO ₃ H'†	α -Amino adipic acid
15			0.18	22.4
30			0.36	40
60	0.08 (1.0)	0.73 (3.2)	0.31	38
120	0.31 (2.2)	1.3 (4.9)	0.52	42.7
240			0.62	38

* Counts attributed to intracellular penicillin N and cephalosporin C were in spots which occupied the same position as the extracellular antibiotics but the configuration of the α -amino adipic acid residue in the intracellular compounds has not been determined.

† The material counted migrated to the same position as authentic δ -(α -amino adipoyl)cysteinylvaline when extracts which had been oxidized with performic acid were subjected to electrophoresis at pH 1.8 but its precise nature remains to be established.

five times that observed after 3 hr. with L- α -amino-[6- ^{14}C]adipic acid.

DISCUSSION

Although the resolution of DL- α -amino adipic acid into its optical antipodes was readily accomplished on a gram scale by treatment of N-chloroacetyl-DL- α -amino adipic acid with hog acylase I (Greenstein *et al.* 1953) this procedure was not easily adapted for the efficient resolution of a few milligrams or less of the DL-amino acid. The oxidation of L- α -amino adipic acid in a Warburg respirometer with L-amino acid oxidase from *Crotalus adamanteus* enabled D- α -amino[^{14}C]adipic acid to be obtained from small amounts (about $10\ \mu\text{moles}$) of the ^{14}C -labelled DL-amino acid with no dilution of radioactivity. So far no enzyme appears to have been shown to metabolize specifically the D-isomer of α -amino adipic acid, although the existence of an enzyme in the *Cephalosporium* sp. for which D- α -amino adipic acid is a substrate may be inferred from the finding of some L- α -amino[^{14}C]adipic acid in mycelium to which D- α -amino[^{14}C]adipic acid had previously been added. However, preparations of mycelium which had been subjected to ultrasonic treatment were able to catalyse a transamination reaction leading from α -oxo adipic acid to L- α -amino adipic acid in high yield. This enabled L- α -amino[^{14}C]adipic acid to be obtained from the α -oxo[^{14}C]adipic acid formed during the preparation of the D-isomer.

The use of L-amino acid oxidase for determinations of the optical configuration of ^{14}C -labelled

α -amino adipic acid enabled determinations to be made with extracts from relatively small amounts of mycelium. The method requires that the labelled α -amino adipic acid be separated from other radioactive substances, or that the amounts of the latter and their behaviour to the enzyme be known, but if these conditions are met the accuracy of the method depends not on the amount of α -amino adipic acid available but only on its total radioactivity. For example 1% of the D-isomer can be detected in $0.2\ \mu\text{g}$. of amino acid of specific radioactivity $1\ \mu\text{C}/\mu\text{mole}$.

Since α -amino adipic acid is formed from acetate and α -oxoglutarate in the *Cephalosporium* sp. it was reasonable to assume that the optical configuration of α -amino adipic labelled with ^{14}C from [^{14}C]acetate would be identical with that of the α -amino adipic acid normally present in the intracellular pool. More than 99% of the radioactive α -amino adipic acid appeared to be the L-isomer. If the intracellular pool of 1 g. of damp-dry mycelium contains $0.19\ \mu\text{mole}$ of α -amino adipic acid (Smith *et al.* 1967) the amount of D- α -amino adipic acid in this pool will therefore be less than $0.002\ \mu\text{mole}$. The conclusion that the intracellular α -amino adipic acid was almost entirely the L-isomer was consistent with the finding that almost all (>97%) of the α -amino adipic acid formed from α -oxo adipic acid in broken mycelium by an aminotransferase had the L-configuration. It is of interest, in this connexion, that DL- α -amino adipic acid will not replace D-glutamic acid in the reactions catalysed by D-alanine-D-glutamate transaminase from *Bacillus subtilis* (Martinez-Carrion & Jenkins, 1965).

The term 'intracellular pool' has been used here in an operational sense. It groups together a variety of substances, including two behaving like penicillin N and cephalosporin C respectively, which escape from the mycelial cells when the latter are treated with 70% ethanol and water. But if it is assumed that all the L- or D- α -aminoadipic acid in this pool is equally available for antibiotic biosynthesis estimates can be made of the labelling of antibiotics to be expected after the addition of α -amino-[^{14}C]adipic acid to mycelial suspensions.

When α -aminoadipic acid enters the mycelium from the extracellular fluid the intracellular pool of this amino acid expands. Suppose that the pool is freely expandable, the rate of synthesis of endogenous α -aminoadipic acid and the rate of metabolism of α -aminoadipic acid in the pool being unchanged.

The amount of α -aminoadipic acid found in the pool of 1 g. of damp-dry mycelium harvested after 72 hr. was $0.19 \mu\text{mole}$ (Smith *et al.* 1967). In the experiment described in Table 1 the amount of L- α -amino-[^{14}C]adipic acid ($2.3 \mu\text{C}/\mu\text{mole}$) which had entered this amount of mycelium after resuspension of the latter in water for 60 min. was $0.55 \mu\text{mole}$. The amount of this labelled α -aminoadipic acid in the mycelium was $0.39 \mu\text{mole}$ (Table 1). Hence the specific radioactivity of α -aminoadipic acid in the expanded pool at this time would be $2.3 \times 0.39 / (0.19 + 0.55)$ or $1.21 \mu\text{C}/\mu\text{mole}$. Similarly, the specific radioactivity of the α -aminoadipic acid in the pool at 180 min. would be $0.91 \mu\text{C}/\mu\text{mole}$.

Between 0 and 60 min. the amount of L- α -amino-[^{14}C]adipic acid which entered the intracellular pool and its concentration in the pool increased at approximately linear rates (Table 1). The calculated specific radioactivity ($Sa \mu\text{C}/\mu\text{mole}$) of the α -aminoadipic acid in the pool at t min. would thus be:

$$\frac{2.3 \times 0.0065t}{0.19 + 0.0092t}$$

The average specific radioactivity during the first 60 min. would then be:

$$\frac{1}{60} \int_0^{60} \frac{2.3 \times 0.0065t}{0.19 + 0.0092t} dt$$

i.e. $0.863 \mu\text{C}/\mu\text{mole}$. In this time $0.55 - 0.39$, i.e. $0.16 \mu\text{mole}$ of α -aminoadipic acid ($2.3 \mu\text{C}/\mu\text{mole}$), is lost from the pool of 1 g. of damp-dry mycelium. Thus $0.368/0.863 \mu\text{mole}$ of α -aminoadipic acid turns over in 60 min. and the amount of α -aminoadipic acid in the endogenous pool ($0.19 \mu\text{mole}$) would turn over in 26.7 min. The rate of synthesis of penicillin N and cephalosporin C was also approximately linear, reaching 0.138 and $0.102 \mu\text{mole/g.}$

of damp-dry mycelium respectively after 60 min. If the α -aminoadipic acid in the pool were incorporated into the antibiotics without further dilution of radioactivity the overall specific radioactivity of the two antibiotics formed in this time would be obtained from

$$\frac{1}{t} \int_0^t Sadt$$

On this basis the specific radioactivity of the penicillin N and cephalosporin C formed during the first 60 min. would be $0.867 \mu\text{C}/\mu\text{mole}$. The corresponding value for the antibiotics formed between 60 and 180 min., when the rate of uptake decreased, would be approximately $1.1 \mu\text{C}/\mu\text{mole}$.

On the assumption that an intracellular pool was present initially which contained $0.03 \mu\text{mole}$ of unlabelled penicillin N and $0.01 \mu\text{mole}$ of unlabelled cephalosporin C/g. of damp-dry mycelium (Smith *et al.* 1967), the specific radioactivities of the intracellular plus extracellular penicillin N and cephalosporin C present after 60 min. would be 0.75 and $0.79 \mu\text{C}/\mu\text{mole}$ respectively, representing a dilution of the specific radioactivity of the α -aminoadipic acid added of 3.05- and 2.92-fold respectively. The specific radioactivities of the extracellular antibiotics after 60 min. would be expected to be lower than those in the intracellular pool. A further decrease in the expected values for the extracellular antibiotics could result from the passage of ^{14}C through intermediates to the antibiotics if the pools of such intermediates reached a significant size.

Since some error may be involved in the estimates of the amounts of intracellular α -aminoadipic acid, the values shown in Table 2 for the dilutions of radioactivity associated with the incorporation of added α -amino-[^{14}C]adipic acid into penicillin N and cephalosporin C do not appear to be inconsistent with the assumption that a substantial proportion (though not necessarily all) of the amino acid which enters the mycelium equilibrates with a pool of endogenous L- α -aminoadipic acid from which the D- α -aminoadipoyl side chains of the antibiotics are derived.

The significance of labelling of penicillin N and cephalosporin C with ^{14}C from D- α -amino-[^{14}C]adipic acid must be considered in relation to the finding that no pool of D- α -aminoadipic acid comparable in size with that of the L-isomer appeared to be formed by endogenous metabolism. The amount, if any, of free D- α -aminoadipic acid/g. of damp-dry mycelium appeared to be less than $0.002 \mu\text{mole}$. The amount of D- α -amino-[^{14}C]adipic acid which had entered the mycelium in 360 min. [corresponding to 66% of the total radioactivity lost from the extracellular fluid plus 5.5% in extra-

cellular antibiotics (Table 2)] was thus large ($0.63 \mu\text{mole}$) compared with that originally present ($< 0.002 \mu\text{mole}$) and also larger than the estimated size of the pools of intracellular antibiotics (Smith *et al.* 1967). Suppose that X is the size of a pool of an amino acid and that an amount dx of the amino acid of specific radioactivity Sa_1 enters the pool and the same amount leaves with specific radioactivity Sa_2 . Then:

$$XdSa_2 = Sa_1 dx - Sa_2 dx$$

$$\frac{dSa_2}{Sa_1 - Sa_2} = \frac{dx}{X}$$

$$\text{or } 2.303 \log \left[\frac{Sa_1}{Sa_1 - Sa_2} \right] = \frac{x}{X}$$

on integration from $Sa_2 = 0$ to $Sa_2 = Sa_1$. Hence, Sa_2 will have reached 90% of the value of Sa_1 when the amount of amino acid which has entered the pool is 2.303 times the amount in the pool, i.e. within 2 min. ($0.63 \times 2.303 / 0.002 \times 360$). The value of Sa_2 will increase less rapidly when the pool is expanding, as it appears to be when D- α -amino[^{14}C]adipic acid enters the mycelium. Nevertheless, the specific radioactivity of the intracellular α -aminoadipic acid would be expected to approach that of the amino acid entering the pool during most of the experiment.

To act as the precursor of the side chains of the antibiotics, at least $1.25 \mu\text{moles}$ of D- α -aminoadipic acid would have been required to pass through the pool in 360 min. If the D-amino acid were formed endogenously at this rate, the dilution of the ^{14}C -labelled amino acid entering the pool would be approximately threefold. If 20% of the D- α -aminoadipic acid were converted into the L-isomer and made unavailable as an antibiotic precursor, the dilution would be increased to about 3.5-fold. In view of the apparently small size of the pools of intracellular antibiotics and other intermediates, incorporation of D- α -aminoadipic acid into the extracellular antibiotics might have been associated with a dilution of specific radioactivity of less than fivefold. The estimated experimental values of approximately 25-fold (Table 2) therefore throw some doubt on the ability of free D- α -aminoadipic acid to function as an intermediate. A similar doubt arises (Table 2) from a consideration of the proportion of added D- α -amino[^{14}C]adipic acid used for antibiotic synthesis (5.5%). Of the amount of α -aminoadipic acid assumed to enter the pool from exogenous and endogenous sources ($3.16 \mu\text{moles/g.}$ of damp-dry mycelium), at least $1.25 \mu\text{moles}$ (40%) would be required for the side chains of penicillin N and cephalosporin C.

If the first step in the use of D- α -amino[^{14}C]adipic acid were its conversion into the L-isomer, about

$0.12 \mu\text{mole}$ of the latter (representing the amount of D-isomer taken up by 1 g. of damp-dry mycelium minus the amount finally present) would have been metabolized in 360 min. (Table 2). Since $1.25 \mu\text{moles}$ of α -aminoadipic acid would be used in the formation of antibiotics and an equal amount, at least, in the formation of lysine, the incorporation of ^{14}C into the antibiotics would be accompanied by at least a 20-fold dilution of specific radioactivity. However, if the value found for the proportion of L- α -amino[^{14}C]adipic acid in the pool after 360 min. (23%) represented the proportion during most of the period of resuspension, the calculated mean specific radioactivity of L- α -aminoadipic acid in the intracellular pool would be significantly greater than the specific radioactivities found for the extracellular antibiotics.

Several possibilities arise in connexion with these results. One is that free D- α -aminoadipic acid is an intermediate but that exogenous D- α -aminoadipic acid does not have free access to the site of synthesis. Another is that an activated form of D- α -aminoadipic acid is an intermediate and can only be formed from an activated form of the L-isomer. A third is that the D-configuration of the antibiotic side-chains only arises after L- α -aminoadipic acid has been incorporated into further intermediates. But in this event the possibility arises that L- α -aminoadipic acid formed from D-isomer does not equilibrate freely with L- α -aminoadipic acid synthesized endogenously from acetate. Determinations of the configuration of α -aminoadipic acid in intracellular α -aminoadipoyl peptides and intracellular antibiotics may throw further light on this question.

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