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The Role of Valine in the Biosynthesis of Penicillin N and Cephalosporin C by a *Cephalosporium* sp.

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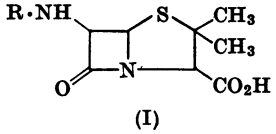
1. The production of penicillin N, but not that of cephalosporin C, was inhibited by the addition of D-valine to suspensions in water of washed mycelium of *Cephalosporium* sp. 8650. The production of cephalosporin C was selectively inhibited by γ-hydroxyvaline. 2. L-[^{14}C]Valine was taken up rapidly and virtually completely by suspensions of washed mycelium but D-[^{14}C]valine and α-oxo[^{14}C]-isovalerate were taken up relatively slowly. 3. Part of the L-valine was rapidly degraded in the mycelium and part was incorporated into protein. Turnover of the valine in the amino acid pool was estimated to occur in 10–17min. 4. No detectable amount of L-[^{14}C]valine was converted into the D-isomer in the mycelium. α-Oxo[^{14}C]isovalerate was rapidly converted into L-[^{14}C]valine in mycelium and mycelial extracts. 5. D-[^{14}C]Valine was partially converted into the L-isomer in the mycelium and ^{14}C from D-valine was incorporated into protein. 6. The labelling of penicillin N and cephalosporin C by ^{14}C from L-[^{14}C]valine was consistent with the view that L-valine is a direct precursor of C_5 fragments of both antibiotics and that any intermediates involved are present in relatively small pools in rapid turnover. 7. Labelling of the antibiotics with ^{14}C from D-[1-^{14}C]valine appeared to occur after the latter had been converted into the L-isomer. Unlabelled D-valine did not decrease the efficiency of incorporation of ^{14}C from L-[1-^{14}C]valine. 8. Intracellular peptide material which contained, among others, residues of α-aminoadipic acid, cysteine and valine, was rapidly labelled by ^{14}C from L-[1-^{14}C]valine in a manner consistent with it being an intermediate in the biosynthesis of one or both of the antibiotics. 9. Labelling of penicillin N from L-[1-^{14}C]valine occurred more rapidly than that of cephalosporin C. However, the effects of D-valine and γ-hydroxyvaline on antibiotic production and the course of labelling of the antibiotics from L-[^{14}C]valine could not readily be explained on the assumption that penicillin N was a precursor of cephalosporin C.

Penicillin N and cephalosporin C are *N*-δ-(D-α-aminoadipoyl) derivatives of 6-aminopenicillanic acid (I, R=H) and 7-aminocephalosporanic acid (II, R=H) respectively. 6-Aminopenicillanic acid can be dissected into residues of L-cysteine and D-valine and 7-aminocephalosporanic acid into residues of L-cysteine and αβ-dehydro-γ-acetoxyvaline.

Studies of the biosynthesis of benzylpenicillin (I, R=$C_6H_5 \cdot CH_2 \cdot CO$) by *Penicillium chrysogenum* have indicated that L-valine is the precursor of the D-penicillamine fragment of the molecule (Demain, 1956; Stevens & de Long, 1958; Arnstein & Margreiter, 1958). The labelling of cephalosporin C by ^{14}C from DL-[1-^{14}C]valine added to fermentations with a *Cephalosporium* sp. have shown that the αβ-dehydro-γ-hydroxyvaline fragment of this antibiotic is derived from valine (Trown, Smith & Abraham, 1963). Subsequent experiments with suspensions of washed mycelium have demonstrated that cephalosporin C can be



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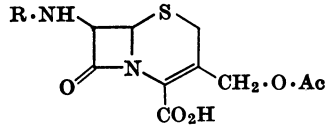

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labelled efficiently by ^{14}C from the L-isomer of valine (Demain, 1963).

This paper is concerned with the relative efficiency of incorporation of ^{14}C from the labelled optical isomers of valine and from α-oxoisovaleric acid into penicillin N and cephalosporin C produced by suspensions of washed mycelium of *Cephalosporium* sp. C.M.I. 49137 (mutant 8650). It also describes the effect of γ-hydroxyvaline and D-valine on antibiotic production and the behaviour of α-oxoisovalerate in cell-free extracts of the mycelium.

MATERIALS AND METHODS

Penicillin N and cephalosporin C were produced in shake flasks by suspensions of the *Cephalosporium* sp. in water under the conditions used by Warren, Newton & Abraham (1967). Antibacterial assays, paper chromatography and electrophoresis, radioautography, the counting of radioactive compounds and the preparation of mycelial fractions were carried out as described by Smith, Warren, Newton & Abraham (1967) and by Warren *et al.* (1967).

The preparations of D- and L-amino acid oxidases used were those described by Smith *et al.* (1967). γ-Hydroxyvaline (mixture of isomers) was synthesized as described by Pollard, Sondheimer & Steward (1958). α-Oxoisovaleric acid was synthesized by the method of Ramage & Simonsen (1935).

Radioactive compounds

D- and L-[1-^{14}C]Valine and α-oxo[1-^{14}C]isovaleric acid. These compounds were prepared from two samples of DL-[1-^{14}C]valine (4·76 μc/μmole and 33·9 μc/μmole respectively) obtained from The Radiochemical Centre, Amersham, Bucks. A solution containing DL-[1-^{14}C]valine (100 μc) in water (0·2ml.) was added to the side arm of a Warburg vessel. With [1-^{14}C]valine of specific radioactivity 33·9 μc/μmole this solution also contained 10·5 μmoles of unlabelled L-valine (when oxidation was carried out with L-amino acid oxidase) or unlabelled D-valine (when D-amino acid oxidase was used). Reaction with the amino acid oxidase was carried out as described by Smith *et al.* (1967). When uptake of O_2 ceased unlabelled L- or D-valine (10 μmoles) in water (0·2ml.) was added from a second side arm and the reaction allowed to proceed until no further uptake of O_2 was observed. The pH of the contents of the flask was then adjusted to 1·3 with HCl and the solution extracted eight to ten times with an equal volume of ether. The ethereal extracts, which contained α-oxo[1-^{14}C]isovaleric acid and about 45% of the total radioactivity used, were combined and stored at −20°. When subjected to electrophoresis on paper at pH4·5 the product from a small sample of the ethereal solution gave one radioactive spot in the same position as authentic α-oxoisovalerate detected by the method of Wagner (1960). For experiments with suspensions of washed mycelium a suitable volume of the ethereal solution was transferred to a flask with a small known volume of 0·06M-sodium phosphate–potassium phosphate buffer, pH7·0, and the ether removed in a rotary evaporator. The resulting solution of sodium α-oxoisovalerate was used immediately.

The aqueous solution remaining after extraction of the α-oxoisovaleric acid with ether was applied to a column (10cm. × 1cm. diam.) of Dowex 50 (X 4; H^+ form; 200–400 mesh) and the column was washed with water. Small amounts of radioactivity in the percolate and washing were associated with a compound which behaved as α-oxoisovalerate on electrophoresis at pH4·5. D- or L-[1-^{14}C]Valine was eluted from the column with N-NH_3, fractions (4ml.) being collected. The radioactive eluate was evaporated to dryness in a rotary evaporator and the residue dissolved in a small known volume of water for further use. Electrophoresis of the product at pH4·5 on paper followed by chromatography in butan-1-ol–acetic acid–water (4:1:4, by vol.) showed that all the radioactivity was in the position of valine. The product had the same specific radioactivity as the starting material (4·76 or 33·9 μc/μmole) and contained 40–45% of the total radioactivity used.

Optical configuration of intracellular valine. This was determined with valine isolated from mycelium 12min. after the addition of L-[1-^{14}C]valine (2·5 μc; 0·0738 μmole) to 5ml. of a suspension of washed mycelium in water (5ml./g. of damp-dry cells) in a 50ml. shake flask. The mycelium was filtered, washed with water (15ml.) and added to ice-cold 70% (v/v) ethanol (5ml.). After the mixture had been kept at 4° for 18hr., the mycelium was separated by filtration and resuspended in water (5ml.) at 4° for 5min. The suspension was filtered and the solid washed with more cold water (5ml.). Ethanol was removed from the first extract by evaporation in a rotary evaporator, the residue combined with the aqueous extract and washings and the resulting solution freeze-dried.

The freeze-dried material from the intracellular pool was dissolved in water (1ml.) and applied to a column (40cm. × 1cm. diam.) of Dowex 50 (X 4; H^+ form; 200–400 mesh). The column was washed with water (100ml.) and a gradient elution carried out by allowing 3N-HCl to enter the reservoir at the head of the column (containing initially 100ml. of water) at a rate of 25ml./hr. (Hirs, Moore & Stein, 1954). Fractions (6·25ml.) were collected every 15min. [^{14}C]-Valine appeared in fractions 32–36. These fractions were combined, evaporated to dryness and the residue was dissolved in water (0·125ml.). The major portion (0·1ml.) of the resulting solution was mixed with a solution of unlabelled DL-valine (20 μmoles) in water (0·1ml.) and placed in one side-arm of a Warburg vessel. Unlabelled DL-valine (20 μmoles) in water (0·2ml.) was placed in the second side-arm. Oxidation with D-amino acid oxidase and determination of the amounts of radioactivity which were subsequently retained on a column (10cm. × 1cm. diam.) of Dowex 50 (X 4) and which appeared in the effluent respectively were carried out as described by Smith *et al.* (1967).

RESULTS

Effect of γ-hydroxyvaline and D- and L-valine on the production of penicillin N and cephalosporin C by suspensions of washed mycelium

γ-Hydroxyvaline. The effect of γ-hydroxyvaline on the production of antibiotics by washed mycelium of the *Cephalosporium* sp. in water is shown in Fig. 1. The yield of cephalosporin C after 6hr. decreased progressively to about 50%

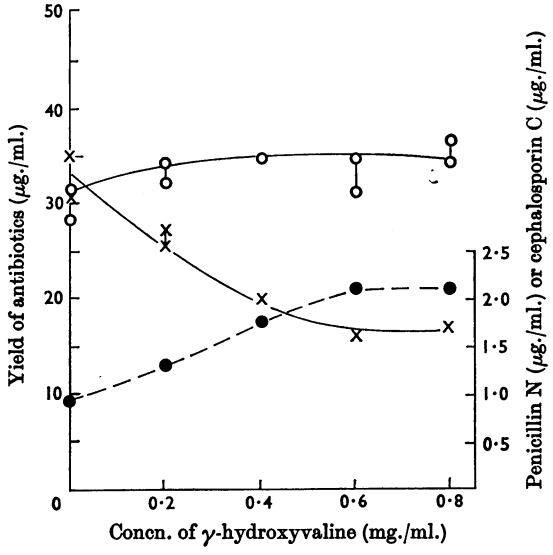

Fig. 1. Production of penicillin N and cephalosporin C after 6hr. at 27·5° in duplicate pairs of 50ml. flasks by shaken suspension of washed mycelium (1g. of damp-dry cells/6ml. of water) containing varying amounts of γ-hydroxyvaline. ○, Penicillin N; ×, cephalosporin C; ●, quotient, penicillin N/cephalosporin C.

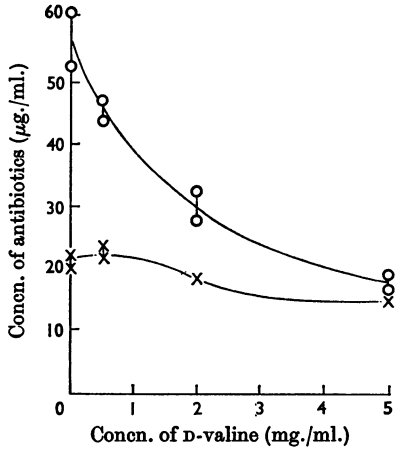

Fig. 2. Production of penicillin N and cephalosporin C after 4hr. at 27·5°, in duplicate pairs of 50ml. flasks by shaken suspensions of washed mycelium (1g./6·6ml. of water) containing varying amounts of D-valine. ○, Penicillin N; ×, cephalosporin C.

of its original value as the concentration of γ-hydroxyvaline was raised to 0·6mg./ml. (4·5mM). In contrast, there was no inhibition of the production of penicillin N and may have been a slight stimulation although in this case the small difference in yield was of doubtful significance.

D- and L-Valine. The effect of D-valine on antibiotic production is shown in Fig. 2. The yield of penicillin N after 4hr. decreased progressively to about one-third of its original value as the concentration of D-valine was raised to 5mg./ml. (43mM). This effect was partially reversed by γ-hydroxyvaline (Table 1), but whether the reversal was caused by an interference with the uptake of D-valine by the mycelium was not determined. The effect of D-valine on the production of cephalosporin C under similar conditions was much less. In the presence of higher concentrations (2–5mg./ml.) the yield of cephalosporin C was decreased by about 25%, but in lower concentrations D-valine appeared to exert a small stimulatory effect in some experiments (Fig. 2).

L-Valine (86 and 117μmoles/ml.) produced no clear-cut change in the yield of penicillin N and cephalosporin C. However, L-valine completely reversed the inhibitory effect of γ-hydroxyvaline on the production of cephalosporin C (Table 1). D-Valine did not reverse this effect of γ-hydroxyvaline.

Uptake and metabolism of D- and L-valine and α-oxoisovaleric acid

Uptake. Fig. 3 shows the change in the radioactivity of the culture fluid with time when given concentrations of D- and L-[1-^{14}C]valine (4·76μc/μmole) and of α-oxo[1-^{14}C]isovalerate (2·79μc/μmole) respectively were added to 25ml. samples of mycelial suspensions (1g. of damp-dry cells/6ml. of water) in 250ml. shake flasks. The uptake of L-[1-^{14}C]valine in the concentration used (0·196μmole/ml.) was nearly complete within less than 10min. A subsequent small rise in the radioactivity of the suspension fluid could be attributed to the excretion of labelled metabolic products from the mycelium. D-[1-^{14}C]Valine and α-oxo[1-^{14}C]isovalerate were taken up much more slowly and at similar rates.

The change in the rate of uptake of L- and D-valine with concentration was determined in shake flasks containing the same amount of labelled amino acid and varying amounts of the corresponding unlabelled amino acid. Lineweaver–Burk plots of $1/v$ against $1/s$, v being the initial rate of uptake and s being the initial concentration of valine, gave straight lines. With L-valine the apparent K_m was 0·03mM and $V_{max.}$ was 0·026μmole/min./ml. With D-valine K_m was 1·15mM and $V_{max.}$ 0·011μmole/min./ml. of suspension.

Metabolism of L-valine by mycelium. Table 2 shows the distribution of ^{14}C in different fractions of the mycelium at varying times after the addition of L-[1-^{14}C]valine (4·76μc/μmole) to suspensions

Table 1. *Effect of γ-hydroxyvaline in the presence of* D- *and* L-*valine respectively on antibiotic production*

Duplicate pairs of 50ml. flasks were used for each mixture of amino acids. Each flask contained 5·2ml. of a suspension of washed mycelium (1g. of damp-dry cells/6·9ml. of water) and amino acids in the concentrations shown. The flasks were shaken at 27·5° and harvested after 4·5hr. The relative yields of penicillin N and cephalosporin C have been derived from the mean assay values for each pair of flasks. Values for individual members of a pair did not differ by more than 10%.

Initial concn. (mg./ml.)			Yield (% of that in the absence of added amino acid)		
γ-Hydroxyvaline	L-Val	D-Val	Penicillin N	Cephalosporin C	Penicillin N / Cephalosporin C
0	0	0	100	100	1·49
0·4	0	0	95	62	2·29
0·4	1·0	0	73	112	0·97
0	1·0	0	87	106	1·22
0·4	0	1·0	75	67	1·66
0	0	1·0	48	100	0·71

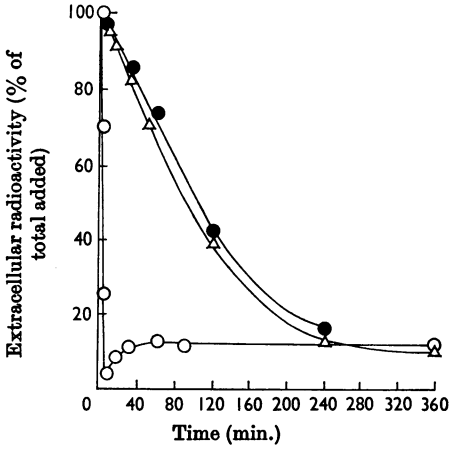

Fig. 3. Uptake of compounds by suspensions of washed mycelium in water shaken at 27·5°. ○, L-Valine; ●, D-valine; △, α-oxoisovalerate. Measurements were made with L-[1-^{14}C]valine (0·196μmole/ml.), D-[1-^{14}C]valine (0·1725μmole/ml.), and α-oxo[1-^{14}C]isovalerate (0·389μmole/ml.).

in shake flasks. The initial concentration of L-[1-^{14}C]valine was 0·1705μmole/ml. Mycelial fractions were obtained as described by Warren *et al.* (1967).

The amount of ^{14}C extractable with aqueous ethanol from the intracellular pool rose rapidly to a maximum in about 8min. as the extracellular concentration of [^{14}C]valine declined. A subsequent fall in the ^{14}C in the intracellular pool was accompanied by the rise in total radioactivity in the hot-trichloroacetic acid, protein and mycelial-residue fractions. However, the total radioactivity recovered from the mycelium was less than that which disappeared from the extracellular fluid and this apparent loss of ^{14}C increased to about 50% within 30min.

In a similar experiment with L-[1-^{14}C]valine (33·9μc/μmole, 0·0147μmole/ml. of suspension), the proportions of the initial radioactivity found in the intracellular pool, hot-trichloroacetic acid extract, 'protein' and mycelial residue were 15·7%, 1·2%, 16·3% and 2·2% respectively after 12min. Since only 11·5% of the added radioactivity remained in the suspension fluid 53·1% of the added isotope was unaccounted for. The [^{14}C]valine isolated from the intracellular pool (see the Materials and Methods section) was at least 99% in the L- configuration.

Evidence that part of the missing ^{14}C in these experiments was in $^{14}CO_2$ was obtained by addition of DL-[1-^{14}C]valine (0·133μmole in 0·2ml. of water) to samples of washed mycelium (0·089g. of damp-dry cells/2ml. of water) in two Warburg vessels. The centre cups contained 3N-KOH. The vessels were gassed with O_2 and shaken at 28°. After 20min. 12·8% and 10·7% respectively, of the isotope originally taken per Warburg vessel (0·625μc), was found in the KOH. It could be assumed that valine taken up by the mycelium was mainly the L-isomer (Fig. 3). Hence about 22% of C-1 of this valine appeared to have been converted into CO_2 under the conditions used.

One hour after the addition of L-[U-^{14}C]valine (4·80μc/μmole) to suspensions of washed mycelium in water in shake flasks (0·755μc/ml.), about 1% of the radioactivity present in the intracellular pool appeared in the position of leucine after electrophoresis on paper at pH 4·5 and chromatography in butan-1-ol–acetic acid–water. Of the radioactivity in the 'protein' fraction of the mycelium 60% appeared in the position of valine and 10% in that of leucine. No radioactive isobutylamine was detected in the intracellular pool, but a small

Table 2. *Distribution of radioactivity in mycelial fractions after addition of* L- *and* D-[^{14}C]*valine and* α-*oxo*[^{14}C]*isovalerate*

The initial concentrations of radioactive compounds in mycelial suspensions were: L-[1-^{14}C]valine, 0·1705 μmole/ml., 0·81 μc/ml.; D-[1-^{14}C]valine, 0·1725 μmole/ml., 0·82 μc/ml.; α-oxo[1-^{14}C]isovalerate, 0·268 μmole/ml., 0·64 μc/ml.; and L-[U-^{14}C]valine, 0·157 μmole/ml., 0·755 μc/ml. (For details see the Materials and Methods section.)

Radioactive compound added	Time (min.)	Radioactivity (% of total added) Hot-trichloroacetic acid extract	Protein	Hydrolysate of mycelial residue
L-[1-^{14}C]Valine	5	0·42	1·8	1·2
	30	0·91	7·3	3·6
	60	1·11	7·1*	3·7
L-[U-^{14}C]Valine	5	1·2	4·5	0·95
	30	2·75	13·0	4·5
	60	1·87	12·4	7·5
D-[1-^{14}C]Valine	5	0·05	0·18	0·38
	30	0·06	0·6	0·43
	60	0·23	2·3	0·8
	120	1·2	2·9	2·25
	240	1·9	7·55	3·55
α-Oxo[1-^{14}C]isovalerate	5	0·23	1·5	0·05
	30	0·21	0·8	0·38
	60	0·44	3·2	1·1
	120	0·98	6·4	5·1
	240	2·42	12·9	6·1

* In other experiments the incorporation into protein at this time approached 15·7%.

amount of radioactivity was found in glutamic acid.

Metabolism of D-*valine.* After addition of D-[1-^{14}C]valine to suspensions of washed mycelium (0·1725 μmole/ml.; 0·82 μc/ml.) the radioactivity of the intracellular pool rose in 120min. to a maximum of about 20% of the total ^{14}C added and thereafter slowly declined. The amounts of radioactivity in the hot-trichloroacetic acid extract, protein and mycelial residue increased slowly with time up to 240min. and after 120min. were 1·2%, 2·9% and 2·25% respectively of the radioactivity added (Table 2). Since about 43% of the initial radioactivity remained in the extracellular fluid after 120min. (Fig. 3) about 24% of the added ^{14}C was not recovered. After 240min. this proportion rose to 60%.

Paper chromatography in butan-1-ol–acetic acid–water, followed by radioautography, indicated that at times up to 240min. a very high proportion of the radioactivity of the intracellular pool was present in valine. The optical configuration of [^{14}C]valine from the intracellular pool was measured 60min. after the addition of D-[1-^{14}C]valine to 5ml. of a suspension of washed mycelium in a shake flask. The initial radioactivity was 0·802 μc/ml. and the concentration of D-valine 0·0236 μmole/ml. The radioactive valine was isolated from the mycelium and treated with D-amino acid oxidase (see the Materials and Methods section). The results indicated that about 60% of the valine was the L-isomer.

Metabolism of α-*oxoisovalerate.* When α-oxo-[1-^{14}C]isovalerate was added to a suspension of washed mycelium (0·268 μmole/ml.; 0·64 μc/ml.) the changes in the distribution of radioactivity in the intracellular pool, hot-trichloroacetic acid extract, protein and mycelial residue resembled those observed with D-[1-^{14}C]valine over a period of 240min. (Table 2). But the maximum amount of radioactivity found in the intracellular pool, after 120min., was only about 12% of the total added. Paper chromatography in butan-1-ol–acetic acid–water, followed by radioautography, showed that the radioactivity of the intracellular pool was distributed between valine and compounds of lower R_F value, but that no detectable amount of α-oxo-[^{14}C]isovaleric acid (R_{Val} 1·45) was present. [^{14}C]-Valine isolated from the intracellular pool after 240min. was found by treatment with D-amino acid oxidase to contain more than 97% of the L-isomer.

Supernatant fluid from a mycelial suspension treated ultrasonically was prepared as described by Warren *et al.* (1967). α-Oxo[1-^{14}C]isovalerate (0·142 μc; 0·06 μmole) was added to 4·3ml. of the supernatant in a 50ml. shake flask and the flask shaken for 3·5hr. at 27°. The contents of the flask were then applied to a column (10cm. × 1cm. diam.)

of Dowex 50 (X 4; H^+ form), the column washed with water (35ml.) and elution carried out with 2N-pyridine (35ml.). The radioactivity in the percolate and eluate was associated with compounds which behaved like α-oxoisovaleric acid and valine respectively when subjected to paper chromatography in butan-1-ol–acetic acid–water. The amounts of radioactivity in these two fractions were 9·3% and 90·7% respectively of the radioactivity added initially. Hence, over 90% of the α-oxoisovaleric acid had been converted into valine under the conditions used. Valine from the eluate was treated with D-amino acid oxidase and the product analysed on a column of Dowex 50 (X 4; H^+ form) under the conditions previously described. The results indicated that more than 97% of the valine was the L-isomer.

Incorporation of ^{14}C from L-[1-^{14}C]valine, D-[1-^{14}C]-valine and α-oxo[1-^{14}C]isovalerate into extracellular penicillin N and cephalosporin C

L-[1-^{14}C]*Valine.* The amounts of penicillin N and cephalosporin C formed by a suspension of washed mycelium were estimated from antibacterial assays of the suspension fluids of three shake flasks (containing no [^{14}C]valine) at times 0, 90, 202 and 360min. respectively. The radioactivities of the two antibiotics produced in the presence of [^{14}C]-valine were determined from counts on paper after samples of a similar suspension fluid had been subjected to electrophoresis and chromatography (Warren *et al.* 1967). Fig. 4 shows the change with time of the estimated specific radioactivities of penicillin N and cephalosporin C formed in the presence of 0·196μmole/ml. of L-[1-^{14}C]valine (0·933μc/ml.). Assay values plotted against time gave smooth curves decreasing in slope in a similar manner for both antibiotics. After 90min. the estimated concentrations of penicillin N and cephalosporin C were 0·10μmole/ml. and 0·051μ-mole/ml. respectively and after 360min. 0·19μ-mole/ml. and 0·10μmole/ml. respectively.

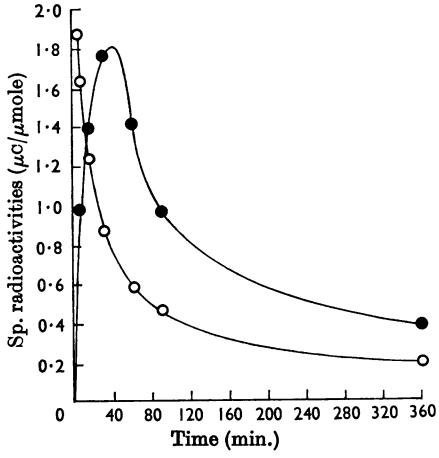

Fig. 4. Estimated specific radioactivities of extracellular penicillin N and cephalosporin C after the addition of L-[1-^{14}C]valine (4·76μc/μmole) to a suspension (25ml.) of washed mycelium in water in a 250ml. shake flask. The initial concentration of L-[1-^{14}C]valine was 0·196μmole/ml. and the initial radioactivity 0·933μc/ml. ○, Penicillin N; ●, cephalosporin C.

The results showed that the specific radioactivity of penicillin N had reached a relatively high value within 4min. of the addition of L-[1-^{14}C]valine, when the first sample was taken. Within 8min. the specific radioactivity of cephalosporin C had reached a relatively high value, but continued to rise and reached a maximum within about 40min. when the value for penicillin N was falling rapidly. During the following 320min. the specific radioactivities of both antibiotics fell progressively, but the values for cephalosporin C remained higher than those for penicillin N. In a comparable experiment with the same sample of L-[1-^{14}C]valine (referred to in Tables 2 and 4) the estimated specific radioactivities of extracellular penicillin N and cephalosporin C after 30min. were 0·93 and 0·7μc/μmole respectively.

Results of an experiment in which L-[1-^{14}C]-valine of higher specific radioactivity (33·9μc/μmole) was added to a suspension of washed mycelium are shown in Fig. 5. The initial concentration of the [^{14}C]valine was 0·048μmole/ml. This suspension produced considerably less than the normal amount of cephalosporin C and much less penicillin N. The yields of extracellular antibiotics increased linearly with time for 30min. and then corresponded to 9·7mμmoles of penicillin N/ml. and 15·4mμmoles of cephalosporin C/ml. in the suspension fluid. The rate of production of both antibiotics subsequently declined and after 60min. the production of penicillin N virtually ceased. The specific radioactivity of extracellular penicillin N rose rapidly with time and reached about 60% of its maximum value within 5min. It showed relatively little change after 30min. As in the experiment with higher concentrations of [1-^{14}C]-valine the apparent specific radioactivity of cephalosporin C within the first 10min. increased much less rapidly than that of penicillin N, but by 40min. it had reached a considerably higher value than that of penicillin N.

Estimates of specific radioactivities of valine in the intracellular pool at different times (Fig. 5) were based on the assumption that the valine pool expanded freely with the entry of the amino acid from the suspension fluid but returned to an

original value of 0·062 μmole/ml. after 30 min. (Smith *et al.* 1967).

D-[1-^{14}C]*Valine*. Table 3 shows the estimated specific radioactivities of penicillin N and cephalosporin C after the addition of two different amounts of D-[1-^{14}C]valine to suspensions of washed mycelium in water. In Expt. 1 the initial concentration of [1-^{14}C]valine (4·75 μc/μmole) in the extracellular fluid was 0·29 μmole/ml. and in Expt. 2 the initial concentration of [1-^{14}C]valine (33·9 μc/μmole) was 0·0256 μmole/ml.

In the two experiments the specific radioactivities of penicillin N and cephalosporin C were higher after 300 min. and 120 min. than after 90 min. and 60 min. respectively. As in the experiments with L-valine the apparent specific radioactivity of cephalosporin C after 60 or 90 min. was significantly greater than that of penicillin N. In the experiment with D-[1-^{14}C]valine of higher specific radioactivity (33·9 μc/μmole) the incorporation of ^{14}C into penicillin N and cephalosporin C was accompanied by dilutions of 1 in 103 and 1 in 61 respectively. Dilutions of the same order, though lower (1 in 25 and 1 in 12 respectively), were found after 60 min. in a comparable experiment with L-[1-^{14}C]valine (Fig. 5).

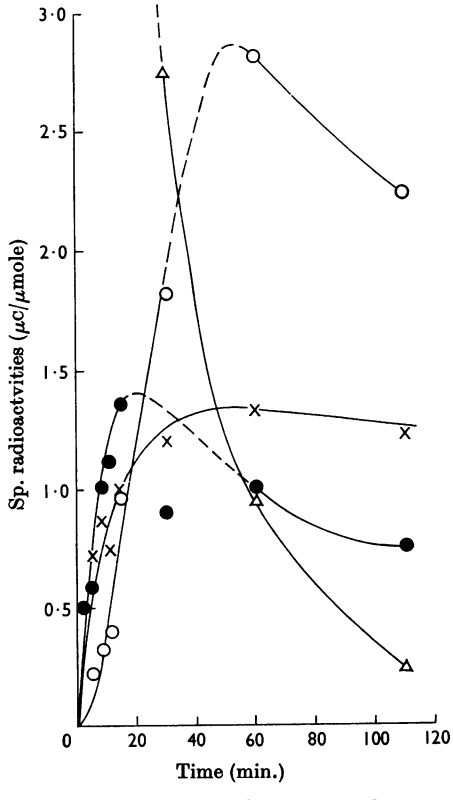

Fig. 5. Estimated specific radioactivites of extracellular penicillin N and cephalosporin C, intracellular valine and intracellular 'ACV' after addition of L-[1-^{14}C]valine (33·9 μc/μmole) to a suspension of washed mycelium. The initial concentration of [1-^{14}C]valine in the extracellular fluid was 0·048 μmole/ml. △, Intracellular valine; ●, intracellular 'ACV'; ×, extracellular penicillin N; ○, extracellular cephalosporin C.

α-Oxo[1-^{14}C]*isovaleric acid*. In a single experiment, in which α-oxo[1-^{14}C]isovalerate (2·61 μc/μmole) was added to a suspension of washed mycelium in water to give an initial concentration 0·268 μmole/ml., the specific radioactivities of extracellular penicillin N and cephalosporin C increased over a period of 240 min. After 120 min. the estimated radioactivities of the two antibiotics were 0·11 μc/μmole and 0·15 μc/μmole respectively. The incorporation of ^{14}C was thus associated with dilutions of 1 in 24 and 1 in 17 respectively. These values are comparable with those found after 90 min. (1 in 35 and 1 in 18 respectively) when D-[1-^{14}C]valine was added to a suspension to give a concentration of 0·29 μmole/ml. (Table 3).

L-[1-^{14}C]*Valine in the presence of unlabelled* D-*valine*. Duplicate samples of the same batch of washed mycelium were suspended in water in two shake flasks (1 g. of damp-dry cells/6 ml. of water). Unlabelled D-valine was added to one flask to give a concentration 17·1 μmoles/ml. and after 60 min. L-[1-^{14}C]valine (4·75 μc/μmole) was added to each flask to give a concentration 0·157 μmole/ml. After a further 20 min. a two-dimensional analysis of the extracellular products was made by electrophoresis and chromatography. The estimated specific radioactivities of penicillin N and cephalosporin C formed in the absence of added D-valine were 0·56 and 0·66 μc/μmole respectively, whereas in the presence of D-valine the corresponding values were 0·59 and 0·60 μc/μmole respectively. There was thus no indication that the added D-valine had competed effectively with L-valine for incorporation into the antibiotics.

Pattern of labelling of extracellular compounds. Radioautography after paper electrophoresis and chromatography of samples of extracellular fluid from suspensions to which L- or D-[1-^{14}C]valine or α-oxo[1-^{14}C]isovalerate had been added revealed only four major radioactive spots. These spots corresponded in position to penicillin N, cephalosporin C, deacetylcephalosporin C and the ^{14}C-labelled precursor respectively. The radioactivity in the position of deacetylcephalosporin C was several-fold lower than that in the position of cephalosporin C itself and reached a detectable level later than the latter. However, since antibacterial assays did not enable the concentration of deacetylcephalosporin C to be determined no estimates could be made of its specific radioactivity.

Table 3. *Incorporation of* ^{14}C *from* D-[1-^{14}C]*valine into penicillin N and cephalosporin C*

In Expt. 1 the initial concentration of D-[1-^{14}C]valine in the extracellular fluid was 0·29 μmole/ml. and its specific radioactivity 4·75 μc/μmole. In Expt. 2 the concentration was 0·0256 μmole/ml. and specific radioactivity 33·9 μc/μmole. Antibiotic concentrations were estimated by antibacterial assays on samples from control flasks containing the same amounts of unlabelled D-valine. For measurements of radioactivity flasks were harvested at the times shown after the addition of isotope.

Expt. no.	Time (min.)	Yield (mμmoles/ml.)		Estimated sp. radioactivity (μc/μmole)	
		Penicillin N	Cephalosporin C	Penicillin N	Cephalosporin C
1	90	47	29	0·134	0·272
	300	130	94	0·183	0·365
2	60	16	19	0·33	0·55
	120	20	27	0·59	0·82

Incorporation of ^{14}C *into mycelial fractions*

Intracellular pool. Radioautography revealed the presence of a number of radioactive substances in the intracellular pool within a few minutes after the addition of either L- or D-[^{14}C]valine or α-oxo-[^{14}C]isovalerate to suspensions of washed mycelium.

One compound corresponded in position to penicillin N on two-dimensional electrophoresis and chromatography on paper. Four compounds with lower R_F values were only partly resolved from each other, and one of them was not completely resolved from added cephalosporin C. Three unidentified radioactive compounds showed no net charge at pH 4·5 and R_{val} values 0·44, 0·53 and 0·63 respectively. A further compound showed an R_F value slightly greater than that of deacetylcephalosporin C, but migrated about 1·5 times as far as the latter towards the anode at pH 4·5. After 30 min. a compound in a position corresponding to deacetylcephalosporin C was also labelled. No radioactivity was found in positions corresponding to 6-aminopenicillanic acid or 7-aminocephalosporanic acid.

After oxidation of material from the intracellular pool with performic acid, electrophoresis on paper at pH 1·8 revealed the presence of three radioactive spots which appeared to be due to sulphonic acids in that they migrated towards the anode at pH 1·8. One occupied the position of penicillaminic acid (β-sulphovaline). Another was due to material which migrated more slowly than glutathione sulphonic acid and was not resolved from the sulphonic acid obtained by oxidation of a sample of synthetic δ-(α-aminoadipoyl)/cysteinylvaline ($ACVSO_3H$). It is referred to subsequently as '$ACVSO_3H$', but its relationship to this tripeptide remains to be established. The third migrated to a position between that of glutathione sulphonic acid and '$ACVSO_3H$'. Acid hydrolysis of material from the intracellular pool which migrated to the same position as $ACVSO_3H$ at pH 1·8 yielded glutamic acid and glycine, as well as α-aminoadipic acid, cysteic acid and [^{14}C]valine, despite the fact that the material had been completely resolved from the sulphonic acid of glutathione.

Table 4 and Fig. 5 show the change with time of the estimated specific radioactivity of intracellular penicillin N and '$ACVSO_3H$' respectively for 110 min. after the addition of L-[1-^{14}C]valine to a mycelial suspension. The values for intracellular penicillin N and '$ACVSO_3H$' were calculated on the assumption that their concentrations in the pool remained constant at 0·026 μmole/g. of damp-dry mycelium (the value estimated for penicillin N by Smith *et al.* 1967). The calculated specific radioactivity of penicillin N rose to a maximum in approximately 15 min. and then slowly declined. '$ACVSO_3H$' was labelled as rapidly as penicillin N, its calculated specific radioactivity reaching a maximum in 15–20 min. and then falling during the subsequent 20 min. The radioactivity of material in the position of cephalosporin C appeared to reach a maximum later than that of penicillin N, but the quantitative significance of counts on this material was made doubtful by the presence of other compounds from which cephalosporin C was not completely resolved.

With D-[1-^{14}C]valine and α-oxo[1-^{14}C]isovalerate the radioactivity of compounds in the intracellular pool increased much more slowly than with L-[1-^{14}C]valine, as was to be expected from the much slower uptake of the former compounds by the mycelium.

Protein and other mycelial fractions. Table 2 shows the relative amounts of radioactivity from L-[1-^{14}C]valine, L-[U-^{14}C]valine, D-[1-^{14}C]valine and α-oxo[1-^{14}C]isovalerate into fractions obtained from the mycelium after extraction of the intracellular pool with 70% (v/v) ethanol (Smith *et al.* 1967). The total radioactivity in the hot trichloroacetic acid extract, protein and an acid hydrolysate of the mycelial residue reached a maximum within

Table 4. *Estimated specific radioactivities of intracellular valine and penicillin N after the addition of* L-[1-^{14}C]*valine to a suspension of washed mycelium*

In Expt. 1 the conditions were those given in Table 2. In Expt. 2 the conditions were those given in Fig. 5. For assumptions concerning the concentrations of valine and antibiotic in the intracellular pool see the text. [^{14}C]Valine denotes valine with the same specific radioactivity as the [^{14}C]valine added. Values given for total intracellular free valine and for the specific radioactivity of this valine are based on the assumption that the pool expanded from a value of 0·062 μmole/ml. with the entry of [^{14}C]valine but fell exponentially to its normal value between 11 and 30 min. Values in parentheses are based on the assumption that the pool size remained at its expanded value.

Expt. no.	Time (min.)	Intracellular amount (μmole/ml. of suspension)		Estimated sp. intracellular radioactivity (μc/μmole)	
		[^{14}C]Valine	Total valine	Valine	Penicillin N
1	5	0·046	0·108	2·03	1·26
	30	0·010	0·062 (0·108)	0·77 (0·44)	0·64
	60	0·002	0·062 (0·108)	0·15 (0·09)	0·23
2	2	0·010	0·072	4·8	0·50
	5	0·015	0·077	6·7	0·59
	8	0·021	0·083	8·6	0·74
	11	0·021	0·083	8·6	0·75
	15	0·016	0·078 (0·083)	7·2 (6·7)	0·8 (approx.)
	30	0·005	0·062 (0·083)	2·7 (2·1)	0·51
	60	0·0018	0·062 (0·083)	1·0 (0·7)	0·11
	110	0·0004	0·062 (0·083)	0·24 (0·18)	0·12

30 min. after the addition of L-[^{14}C]valine, but continued to increase in most cases up to 240 min. after the addition of D-[1-^{14}C]valine.

In a further experiment with L-[1-^{14}C]valine (Fig. 5) the quotient (total ^{14}C in protein)/(total ^{14}C in antibiotics) 60 min. after the addition of the isotope was 4·6. With D-[1-^{14}C]valine of the same specific radioactivity (Table 3) the corresponding value was 4·0.

DISCUSSION

The structure of cephalosporin C suggested that its biosynthesis might involve the oxidation of valine to γ-hydroxyvaline and incorporation of the latter into the C_5 fragment of the β-lactam dihydrothiazine ring system. The depression of the production of cephalosporin C by a mixture of the optical isomers of γ-hydroxyvaline gave no support to this hypothesis, although it is conceivable that the utilization of one of the isomers was depressed by others.

The inhibition of the production of penicillin N by D-valine is consistent with the view that L-valine is the precursor of the D-penicillamine fragment of the penicillin molecule in the *Cephalosporium* sp, as it appears to be with benzylpenicillin in *Penicillium chrysogenum* (Demain, 1956; Stevens & de Long, 1958; Arnstein & Margreiter, 1958). The mode of action of D-valine has not been established, but its failure to inhibit significantly the production of cephalosporin C suggests that it affects either a step in the biosynthetic pathway to penicillin N, which occurs after this pathway has diverged from that leading to cephalosporin C, or a step which is rate-limiting only for penicillin N, or both steps.

An interpretation of the results obtained when ^{14}C-labelled precursors were added to a suspension of washed mycelium requires knowledge of the uptake of these precursors by the mycelium and their dilution with endogenous precursors in the cell. Under the conditions used, L-[^{14}C]valine was taken up virtually completely within 5–10 min., presumably by a process of active transport, and no significant amount (< 2%) of this amino acid was converted in the mycelium into the D-isomer. The amount of the added L-[^{14}C]valine in the intracellular pool reached a maximum when uptake was complete, but fell rapidly over the next 20 min. Some of this valine was incorporated into protein, but a considerable proportion was degraded to carbon dioxide and unidentified compounds. No radioactive isobutylamine was detected in the mycelium after the addition of L-[U-^{14}C]-valine and the radioactivity not recovered was greater than could be accounted for by the loss of C-1 as $^{14}CO_2$ during the conversion of some of the valine into leucine (Abelson, 1954). Possibly L-valine was metabolized, via isobutyrate and propionate, to succinylcoenzyme A (Greenberg, 1961; Sokatch, 1964).

To calculate the specific radioactivities of valine in the amino acid pool from the total radioactivity of this valine it was necessary to make assumptions about the size of the pool and its change in size

with time after the valine had entered the mycelium from the extracellular fluid. It has been assumed here that the rate of metabolism of valine did not change significantly during the rapid uptake of L-[^{14}C]valine, so that the amount of valine in the pool increased by an amount corresponding to: radioactivity in intracellular valine (μc)/sp. radioactivity of added valine (μc/μmole). The values given in Table 4 and Fig. 5 are based on the further assumption that the amount of valine in the pool then fell exponentially to its original value by 30min. The values would have been up to 25% lower at this time if the subsequent fall had not occurred. However, a possibility of more serious error arises in relation to the value assumed for the amount of valine normally present in the pool (0·062 μmole/ml. of suspension). Although this value was obtained from one series of experiments with mycelium harvested 72hr. after inoculation (Smith *et al.* 1967), significantly higher values were obtained in this experiment after 120hr. with the same batch of mycelium and in some later experiments after 72hr.

If the total amount of valine in the pool remains constant at X μmoles/ml. of suspension, of which x μmoles/ml. is [^{14}C]valine with the same specific radioactivity as that of the valine added to the suspension, then $X\mathrm{d}x/x = k\mathrm{d}t$, where k is the rate (μmoles/min.) at which valine enters and leaves the pool. Hence $k = 2{\cdot}303X[\log x]_{x_{t1}}^{x_{t2}}(t_2 - t_1)$. Substitution in this equation of the maximum value of X obtained from the data used for Expt. 2 in Table 4 (0·083 μmole/ml. of suspension at 8–11min.) and the measured values of x for the periods 11–15min. and 15–30min. gave $k = 0{\cdot}0046$ and $k = 0{\cdot}0062$ μmole/ml./min. respectively. Hence, the estimated times of turnover of the original valine pool were 13·5 and 10min. respectively. On the assumption that the pool size had fallen to its original value by 30min. the calculated turnover time, from measurements made at 30 and 60min., was 17min.

In Expt. 1 of Table 4 the calculated mean specific radioactivity of the valine in the intracellular pool during the first 30min. was about 1·3 μc/μmole. Since the corresponding values for extracellular penicillin N and cephalosporin C were 0·93 and 0·7 μc/μmole respectively it appeared that the pools of intermediates between valine and the extracellular antibiotics were relatively small and in rapid turnover. This also appeared to be so from the estimated specific radioactivity of intracellular penicillin N.

In the experiment of Fig. 5 (also Expt. 2 of Table 4) the mean specific radioactivity of the intracellular valine during the first 30min. was about 5·9. Since the estimated specific radioactivities of penicillin N and cephalosporin C were 1·2 and 1·8 μc/μmole respectively at 30min., the incorporation of ^{14}C into the antibiotics appeared to be associated with a greater dilution than was the case in Expt. 1. This may be associated with the fact that the unusually small amounts of extracellular antibiotics formed in 30min. in this experiment (0·0097 μmole/ml. of penicillin N and 0·0154 μmole/ml. of cephalosporin C) were not very much greater than the amounts of antibiotics (about 0·005 μmole of penicillin N and 0·002 μmole of cephalosporin C) previously estimated to be present in the intracellular pool from a corresponding amount of mycelium harvested at 72hr. The ^{14}C entering extracellular antibiotics might thus have been diluted considerably by the intracellular pools until after the specific radioactivity of the intracellular valine had fallen from its maximum value.

In one experiment with L-[1-^{14}C]valine the specific radioactivity of extracellular penicillin N clearly reached a higher maximum and rose to its maximum earlier than that of cephalosporin C (Fig. 4). This would be expected if the biosynthesis of cephalosporin C involved more intermediates reaching significant concentrations than did that of penicillin N and thus raised the question whether cephalosporin C was formed from penicillin N. In a second experiment (Fig. 5) extracellular penicillin N was also labelled more rapidly than cephalosporin C in the first 15min., but the situation was subsequently complicated by the decrease in the rate of synthesis of both antibiotics and virtual cessation of that of penicillin N. The intracellular peptide material described as 'ACV' was labelled more rapidly, in the first 15min., than extracellular penicillin N, and reached its maximum specific radioactivity approximately as soon as intracellular penicillin N. This would be consistent with the view that one component, at least, of the peptide material was an intermediate in the biosynthesis of this antibiotic. A subsequent fall and rise in specific radioactivity would have appeared if the smooth curve for 'ACV' in Fig. 5 had been drawn through the point corresponding to the value at 30min. The possibility therefore arises that the material contains two components labelled at different rates.

If both antibiotics had been synthesized at constant rates throughout the experiment of Fig. 5 the estimated value for the maximum specific radioactivity of cephalosporin C, which was higher than that of penicillin N, would have been inconsistent with the hypothesis that the latter is a precursor of the former. Similarly, if penicillin N were the immediate precursor of cephalosporin C the specific radioactivity of the latter should not have continued to increase, as shown in Fig. 4, after it had reached the specific radioactivity of

the former. A precursor–product relationship between penicillin N and cephalosporin C could not in fact be rigidly excluded from the results of this experiment. If intracellular penicillin N had continued to be formed and converted into cephalosporin C after a drastic decrease in the rate of appearance of penicillin N in the extracellular fluid, the specific radioactivity of extracellular cephalosporin C could have risen above that of penicillin N. In experiments in which α-amino-L-[^{14}C]adipic acid (Warren *et al.* 1967) and D-[1-^{14}C]valine respectively were used as precursors, the apparent values for the specific radioactivity of cephalosporin C were also higher than those of penicillin N. This raises the question whether the radioactive spot in the cephalosporin C position after paper electrophoresis and chromatography was unresolved from a different compound, such as the Δ^2-isomer, with no antibacterial activity, or whether some other undetected error was involved in estimates of the specific radioactivities of one of the antibiotics from measurements of antibacterial activities and subsequent counts of radioactivity on paper. If, however, the observed differences between the specific radioactivities of penicillin N and those of cephalosporin C are correct, the possibility must be considered that the two antibiotics are not formed from a common pool of precursors (L-valine and L-α-aminoadipic acid) at the same site within the cell.

The hypothesis that cephalosporin C is formed from penicillin N is a plausible one on chemical grounds (Morin *et al.* 1963). Nevertheless, the course of labelling of the antibiotics from L-[^{14}C]-valine added no reliable support to this hypothesis. Moreover, the hypothesis did not easily account for the findings that D-valine selectively depressed the production of penicillin N and that γ-hydroxyvaline depressed the production of cephalosporin C without also causing a comparable increase in that of penicillin N.

α-Oxoisovalerate was rapidly converted into L-valine in the mycelium and in mycelial extracts. The relatively slow rate of uptake of α-oxo[1-^{14}C]-isovalerate from the extracellular fluid was presumably responsible for the gradual rise in the specific radioactivity of penicillin N and cephalosporin C when this substance was added to suspensions of washed mycelium. A similar slow rise in the specific activity of the antibiotics occurred after the addition of D-[1-^{14}C]valine (Table 3). Estimation of the optical configuration of the radioactive amino acid in the intracellular pool and the incorporation of ^{14}C into protein indicated that a substantial proportion of the D-valine which slowly entered the mycelium was converted into the L-isomer. In one experiment (described in Table 2), in which the amount of added D-[1-^{14}C]valine was 0·173 μmole/ml., the amount of radioactive valine found in the intracellular pool after 60 min. was about 0·014 μmole/ml. of suspension and about 60% of this was the L-isomer. On the assumption that the normal pool of L-valine had expanded by an amount equal to the amount of radioactive L-valine which it contained, the specific radioactivity of L-valine in the pool at this time would be 0·57 μc/μmole. In a comparable experiment in which the amount of added D-[1-^{14}C]valine was 0·29 μmole/ml. (Table 3) a proportionally higher value of about 1·1 μc/μmole would have been expected. Thus the estimated mean specific radioactivity of the L-valine in the pool during 60 min. would have been 0·55 μc/μmole. The estimated specific radioactivities of extracellular penicillin N and cephalosporin C after 90 min. were 0·134 and 0·272 μc/μmole respectively and presumably at least two-thirds as high after 60 min. Hence the labelling of the antibiotics after the addition of D-[1-^{14}C]valine indicated that the D-valine was converted into the L-isomer and that the latter was then used as a precursor. The failure of added unlabelled D-valine to depress significantly the specific radioactivities of antibiotics formed in the presence of added L-[1-^{14}C]valine was consistent with this view.

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