The Biosynthesis of Penicillin

5. COMPARISON OF VALINE AND HYDROXYVALINE AS PENICILLIN PRECURSORS*

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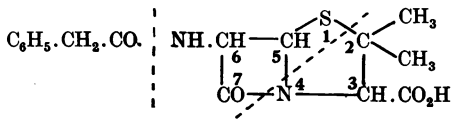
Both cystine (Arnstein & Grant, 1954*a*, *b*; Stevens, Vohra, Inamine & Roholt, 1953) and valine (Arnstein & Grant, 1954*a*; Stevens, Vohra & De Long, 1954) are utilized by *Penicillium chrysogenum* for penicillin biosynthesis, and it has been shown that L-cystine is incorporated intact, presumably after reduction to cysteine, as a precursor of the sulphur atom, the β-lactam carbon atoms ($C_{(5)}$–$C_{(7)}$) and the side-chain nitrogen atom of benzylpenicillin (I) (Arnstein & Grant, 1954*b*). The incorporation of valine, however, was inferred only from experiments with ^{14}C-labelled DL-valine, and no direct utilization of the intact amino acid has been demonstrated.



(I)

In the present work, the biosynthesis of penicillin from valine has therefore been investigated in more detail with DL-valine labelled with both ^{14}C and ^{15}N. Penicillin biosynthesis has also been studied by incubating D- and L-[*carboxy*-^{14}C]valine with washed mycelium for 1–4 hr., as it seemed possible that differences in the utilization of the D- and L-enantiomorphs might be more easily demonstrated in such short-term experiments than in complete fermentations. In view of the D-configuration of the α-carbon atom of naturally occurring penicillamine it was expected that D-valine would be preferentially utilized. It was found, however, that both D- and L-valine were converted into penicillin to an equal extent, provided that allowance was made for differences in uptake of the two forms by the mycelial cells. Experiments are also described which exclude β-hydroxyvaline as an intermediate in the conversion of valine into penicillin. Some of this work has been briefly reported at the 341st meeting of the Biochemical Society (Arnstein & Clubb, 1955).

* Part 4: Halliday & Arnstein (1956).

EXPERIMENTAL

Fermentations

Organism and preparation of cultures. In the present work strains WIS 48–701 and WIS 51–20 of *Penicillium chrysogenum* were used, the latter giving somewhat better yields (up to 550 i.u./ml.) under our experimental conditions. Subcultures and spore inocula were prepared as described previously (Arnstein & Grant, 1954*b*).

Details of medium and fermentations. All fermentations were carried out in a stirred aerated jar fermenter containing 1–1·5 l. of synthetic medium under conditions identical with those used earlier (Arnstein & Grant, 1954*b*), except that with strain WIS 51–20 potassium or ammonium phenylacetate was added every 24 hr., beginning about 40 hr. after inoculation to give a final concentration of 0·1 % instead of 0·2 %.

Penicillin production and purity of the isolated penicillin *N*-ethylpiperidine salt were determined by cup-plate bioassay with *Bacillus subtilis* (I.C.I. strain, NCTC 8241) as test organism (Humphrey & Lightbown, 1952), and international standard sodium benzylpenicillin (1670 i.u./mg.) or its *N*-ethylpiperidine salt as the appropriate standard. Further details of individual fermentations are given in Table 1.

Washed mycelium experiments. Washed mycelium of *P. chrysogenum* WIS 51–20, prepared as described by Halliday & Arnstein (1956), was used at a concentration of 0·1 g. (wet wt.)/ml. of medium, the dry wt. being 10–15 % of the wet wt. The mycelium and incubation medium, which contained 0·005 % potassium phenylacetate and D- or L-[*carboxy*-^{14}C]-valine (50 or 200 μg./ml.), were incubated in conical flasks (250 ml. or 500 ml. for 25 ml. and 50 ml. of medium respectively) at 24° on a rotary shaker (250 rev./min.). Carbon dioxide was collected as $BaCO_3$ in some experiments by incubating the washed mycelium in a closed flask in an atmosphere of O_2 and passing CO_2-free air through the medium and through a trap containing saturated aqueous $Ba(OH)_2$ at the end of the incubation. At the end of the experiment, the mycelium was filtered off and washed with 0·1 M phosphate buffer (pH 6·9). The filtrate and washings were diluted to a known volume and assayed for penicillin activity. Carrier valine (approx. 40 mg./flask) and sodium benzylpenicillin (approx. 40 mg./flask) were added to the solution and penicillin was isolated in the usual way by extraction with ether at pH 2 and conversion into the *N*-ethylpiperidine salt, which was recrystallized to constant radioactivity. The aqueous solution remaining after ether

extraction was brought to pH 7 and evaporated to dryness *in vacuo*. The residue was dissolved in water and valine was isolated by adsorption on Zeo-Karb 215 ion-exchange resin (Permutit Co. Ltd., London) (approx. 5–10 g.) followed by elution with 0·2 M-NH_3 soln. The valine obtained by evaporation of the eleunt was recrystallized to constant radioactivity. The purity of the recrystallized product was checked by paper chromatography.

The mycelium from each flask was further washed with water, dried at room temperature over P_2O_5 and assayed directly for radioactivity.

Isolation and degradation methods

Penicillin. At the end of the fermentation, usually 120–140 hr. after inoculation, benzylpenicillin was isolated from the cooled, filtered broth by ether extraction at pH 2 and concentrated by successive partitioning between ether and buffer (pH 7) at 4° (Smith & Hockenhull, 1952). Finally, the *N*-ethylpiperidine salt was prepared and recrystallized from chloroform–acetone till the radioactivity remained unchanged.

Penicillin was degraded by heating with 0·1 N-H_2SO_4 at 100° essentially as described earlier (Arnstein & Grant, 1954*a*, *b*), except that conversion of the *N*-ethylpiperidine salt into the sodium salt was found to be unnecessary and was omitted. In addition, yields of *iso*propylidenepenicillamine were improved considerably by evaporating the solution of penicillamine hydrochloride, obtained from the mercury mercaptide derivative by treatment with H_2S, by freeze-drying rather than by vacuum distillation. With these two modifications it was possible to isolate pure *iso*propylidenepenicillamine (m.p. 191°) and penilloaldehyde 2:4-dinitrophenylhydrazone (m.p. 195–196°) from as little as 150 mg. of the *N*-ethylpiperidine salt of benzylpenicillin.

For ^{15}N analysis of the penilloaldehyde, dilution with extraneous nitrogen was minimized by preparing the *p*-bromophenylhydrazone derivative instead of the 2:4-dinitrophenylhydrazone. *p*-Bromophenylhydrazine hydrochloride (1%, w/v) in 2 N-HCl was added directly to the solution of penilloaldehyde obtained by degradation of penicillin with acid and $HgCl_2$, excess of the latter having been removed with H_2S. The product was filtered off, washed with water and dried (yield, 36%). After recrystallization from benzene, *penilloaldehyde* p-*bromophenylhydrazone*, m.p. 137°, was obtained. (Found: C, 55·4; H, 4·5; N, 12·1; Br, 22·7; $C_{16}H_{16}ON_3Br$ requires C, 55·5; H, 4·7; N, 12·1; Br, 23·1%.)

Penicillamine and valine. The direct oxidative decarboxylation of either penicillamine disulphide or penicillaminic acid with ninhydrin or chloramine T did not give good yields of the corresponding aldehydes, although the amount of CO_2 was satisfactory. Desulphurization of *iso*propylidenepenicillamine to valine, followed by treatment with ninhydrin, was, however, successful. *iso*Propylidenepenicillamine (50 mg.) in water (5 ml.) was boiled under reflux for 10 min. with 0·5 ml. of a suspension of Raney nickel in water (Mozingo, Wolf, Harris & Folkers, 1943). The reaction mixture was cooled, filtered and evaporated to dryness. In trial experiments, valine was found to be the only amino acid present, as shown by paper chromatography with two solvents [phenol–water, 5:2 (v/v) (with NH_3) and amyl alcohol–pyridine–water, 7:7:6 (by vol.)]. The above residue was therefore dissolved in 10 ml. of M potassium phosphate buffer (pH 5·8) and the solution was boiled briefly while CO_2-free N_2 was passed through. Ninhydrin (100 mg.) was then added and the solution boiled for a further 45 min. *iso*Butyraldehyde and CO_2 were collected in traps containing a saturated solution of 2:4-dinitrophenylhydrazine in 2 N-HCl and saturated $Ba(OH)_2$ respectively, and isolated as *iso*butyraldehyde 2:4-dinitrophenylhydrazone, m.p. 179–180° (89 mg., 73%) and $BaCO_3$ (17 mg., 40%).

Valine was oxidatively degraded by decarboxylation with either ninhydrin or chloramine T.

Amino acids from mycelial protein. The dried mycelium was defatted by boiling with ethanol and ether. The fat-free product was extracted with 5% (w/v) trichloroacetic acid (25 ml./g.) for 30 min. at room temp., 15 min. at 90° and again 30 min. at room temp. The residue was washed with ethanol and ether and dried at 100°. A portion of the dried protein-rich residue (5 g.) was hydrolysed with 6 N-HCl (50 ml.) at 100–105° for approx. 18 hr. in a sealed tube. The filtered hydrolysate was evaporated to dryness *in vacuo*, the residue dissolved in water (approx. 50 ml.), and the solution neutralized with Na_2CO_3. The amino acids were precipitated by adding alternately small portions of 10% Na_2CO_3 and 25% mercuric acetate (cf. Neuberg & Kerb, 1912). The free amino acids were regenerated by suspending the ppt. in dil. HCl and passing H_2S. The mixed amino acids were chromatographed on a column (60 cm. × 3 cm.) of Dowex 50 or Zeo-Karb 225, which was eluted with 1·5 N- and 2·5 N-HCl (cf. Stein & Moore, 1949). The appropriate fractions were evaporated to dryness and the separated amino acids were adsorbed on short columns of Zeo-Karb 215 resin (H form; approx. 1 g. of resin/10 mg. of N). The columns were washed with water until the effluent was neutral and the amino acids were eluted with 0·2 M-NH_3 soln. After evaporation of the solution to dryness, the amino acids were crystallized from aqueous ethanol and recrystallized to constant radioactivity. The purity of each amino acid was checked by paper chromatography.

Isotope estimations

Radioactivity measurements. Compounds were mounted on 1 cm.[2] polythene disks and counted as samples of 'infinite thickness', a thin end-window Geiger–Müller tube (Popják, 1950) being used. All counts were compared with a poly[^{14}C]methyl methacrylate sheet reference standard obtained from the Radiochemical Centre, Amersham. The standard error in all determinations is less than 5%.

Determination of ^{15}N. Samples were assayed as in previous work (Arnstein & Grant, 1954*b*) with a 60° mass spectrometer. The accuracy of ^{15}N estimations is ±0·003 atom % excess.

Labelled compounds

Uniformly labelled L-[^{14}C]*valine*, DL-[α-^{14}C]*valine and* DL-[carboxy-^{14}C]*valine*. These compounds were obtained from the Radiochemical Centre, Amersham. Uniformly labelled valine will be abbreviated [U-^{14}C]valine in this paper.

Resolution of DL-[carboxy-^{14}C]*valine.* DL-[*carboxy*-^{14}C]-Valine (6·3 mg., 0·1 mc) was diluted with inactive DL-valine (3 g.), converted into the *N*-formyl derivative in 74% yield by formylation with formic acid–acetic anhydride (du Vigneaud & Patterson, 1935), and resolved as the brucine salt (Fischer, 1906). The molecular rotation of the L-[*carboxy*-^{14}C]valine was +61·6±2° (*c* = 0·604 in acetic acid), that of D-[*carboxy*-^{14}C]valine was −55·2±2° (*c* = 0·604 in acetic acid). Under the same conditions, inactive L- and D-valine

obtained from Roche Products Ltd. had identical molecular rotations, but the best literature value is somewhat higher (72·6°; Greenstein, 1954).

DL-[^{15}N]*Valine*. The synthesis of [^{15}N]valine was essentially similar to that used by Adams & Tolbert (1952) for preparing [α-^{14}C]valine. Hippuric acid was prepared in 94% yield from [^{15}N]glycine (4·1 g., 16 atom % excess ^{15}N) by the method of Steiger (1944) and converted into 2-phenyl-4-*iso*propylidene-5-ox[^{15}N]azolone (Cornforth, 1949) in 64% yield. Reduction of the oxazolone (4·64 g.) with P–HI (cf. Adams & Tolbert, 1952) yielded valine, which was purified by adsorption on Zeo-Karb 215 resin (H form, 1 g./10 mg. of N) and elution with 0·2 M-NH_3 soln. after the column had been well washed with water. The alkaline solution was evaporated to dryness *in vacuo* and DL-[^{15}N]valine was crystallized from aqueous ethanol (1·87 g., 66%; overall yield from glycine, 40%).

DL-*β-Hydroxy*[carboxy-^{14}C]*valine*. A solution of *iso*butylmagnesium bromide (0·25 mole) in ether (820 ml.) was allowed to react with $^{14}CO_2$ generated from $Ba^{14}CO_3$ (50 g.; 223·8 μc/mole), as in the method given by Calvin, Heidelberger, Reid, Tolbert & Yankwich (1949). At the end of the reaction the mixture was poured on crushed ice, and a cooled solution of conc. H_2SO_4 (30 ml.) in water (250 ml.) was added with stirring. The aqueous layer was saturated with NaCl, the ether was separated and the aqueous solution was extracted twice with ether (200 ml.). The combined ether solutions were washed twice with saturated NaCl (100 ml.), dried (Na_2SO_4) and evaporated. The residue was distilled at atmospheric pressure, giving [*carboxy*-^{14}C]*iso*valeric acid (11·9 g.; 47·5%).

The *iso*valeric acid (10·8 g.) was brominated in the presence of dry red P (1·32 g.) by the slow addition of Br_2 (12 ml.) at room temp. Finally, the reaction mixture was heated on the steam bath for 24 hr. and then added dropwise to hot water (40 ml.). After heating on the steam bath for 1 hr., it was cooled and extracted three times with ether (2 × 25 ml.; 10 ml.). The ether extracts were combined, washed with saturated NaCl, dried (Na_2SO_4) and evaporated. The residue was distilled at 25 mm., giving 16·1 g. (89%) of crude α-bromo[*carboxy*-^{14}C]*iso*valeric acid.

The crude acid was refluxed with 3 N sodium methoxide in methanol (128 ml.) for 6 hr. (cf. Owen, 1949). Water (300 ml.) was added to the cold solution and methanol was removed by distillation *in vacuo*. The solution was acidified with conc. HCl and extracted with ether. After evaporation of the ether, the oily residue was crystallized by adding water. Yield of $\beta\beta$-dimethyl[*carboxy*-^{14}C]acrylic acid, m.p. 69°, 4·1 g. (46·5%; specific radioactivity 203·2 μc/mole).

$\beta\beta$-Dimethyl[*carboxy*-^{14}C]acrylic acid (4 g.) was converted into α-bromo-β-methoxy[*carboxy*-^{14}C]*iso*valeric acid as described by Rufenach (1952). Yield after recrystallization from light petroleum (b.p. 60–80°) 5·2 g. (62%); m.p. 75–78°; specific radioactivity 225·8 μc/mole.

The product was aminated by heating at 100° for 2 hr. with conc. NH_3 soln. (56 ml., sp.gr. 0·88) in a sealed tube. The solution was evaporated to dryness and the residue was redissolved in hot water (50 ml.). After filtration, ethanol (200 ml.) was added, when α-amino-β-methoxy[*carboxy*-^{14}C]*iso*valeric acid (β-methoxyvaline) crystallized. The product (2 g., 56%, m.p. about 260°) contained no halogen and was found to be chromatographically pure [Whatman no. 3 MM paper with phenol–water, 5:2 (v/v) (with NH_3) as solvent].

Hydrolysis of α-amino-β-methoxy*iso*valeric acid with HBr (Abderhalden & Heyns, 1934) for 1 hr. or more was accompanied by appreciable destruction of the product, β-hydroxyvaline, but 0·5 hr. was found to be sufficient for complete hydrolysis. β-Methoxy[*carboxy*-^{14}C]valine (2 g.) was therefore refluxed for 0·5 hr. with 48% HBr (20 ml.), water was added and HBr removed by evaporation *in vacuo*. The dry residue was dissolved in water and the solution passed through a column of Zeo-Karb 215 resin (50 g., H form). The column was washed with water till the pH of the effluent was neutral and then eluted with 0·2 M-NH_3 soln. The eluate was evaporated to dryness and the residue was

Table 1. *Experimental details of fermentations*

Solutions of the labelled amino acids in about 6 ml. of water were sterilized by Seitz filtration and added in approximately equal portions at the stated times after inoculation of the medium. The amount of penicillin is that present at the end of the fermentation, which was usually the maximum synthesized. *Penicillium chrysogenum* WIS 48–701 was used in Expts. 2, 3, 5 and 6, strain WIS 51–20 in the other experiments. The volume of the fermentation was either 1 l. (Expts. 1–3 and 9) or 1·5 l. (Expts. 4–8 and 10).

	Labelled precursor added						
			Isotope content				
Expt.	Amino acid	Total wt. (mg.)	(μc of ^{14}C/mole)	(atom % excess ^{15}N)	Times of addition of precursor (hr.)	Duration of fermentation (hr.)	Yield of penicillin (i.u./ml.)
1	DL-[α-^{14}C:^{15}N]Valine	224	223	4·63	0, 22, 89, 118, 142	167	248
2		281	510	4·34	0, 25, 46, 65, 94, 113	120	237
3		175	413	4·43	40, 63, 87	153	112
4		348	355	4·50	41, 65, 89	115	428
5		439	339	4·65	45, 67, 91	114	120
6		285	99·6	4·70	46, 96	120	105
7		330	127	4·70	72, 100, 118	160	206
8	L-[U-^{14}C]Valine + DL-[^{15}N]valine*	439	1830	15·52	41, 65, 88, 112	117	421
9	DL-β-Hydroxy[*carboxy*-^{14}C]valine	285	197	—	42, 65, 88, 112	112	147
10		497	197	—	46, 70, 93, 116	134	323

* The mixture contained 0·4 mg. of L-[U-^{14}C]valine (28·5 μc/mg.) and 439 mg. of DL-[^{15}N]valine.

crystallized from aqueous ethanol, giving 1·19 g. (65%) of β-hydroxy[*carboxy*-^{14}C]valine, m.p. 220°, with decomposition. The specific radioactivity was 197·2 μc/mole and the overall yield from $^{14}CO_2$ was 4%. The purity of the product was checked by paper chromatography [Whatman no. 3 MM paper; phenol–water, 5:2 (v/v) (with NH_3)], and by constant radioactivity after recrystallization from aqueous ethanol.

RESULTS

Utilization of valine for biosynthesis of penicillin and mycelial protein

The intact utilization of the carbon chain of valine for the biosynthesis of the penicillamine moiety of penicillin [see formula (I)] is indicated by the distribution of ^{14}C in the mycelial valine and in the penicillin isolated from a fermentation to which L-[U-^{14}C]valine had been added (Table 2). As in the experiments with DL-[α-^{14}C]valine (cf. Table 6), nearly all the radioactivity (95%) in the penicillin was located in the penicillamine portion of the molecule. Further degradation of penicillamine showed that the average radioactivity per carbon atom (6·5 μc) was almost identical with the average radioactivity of $C_{(2)}$, $C_{(3)}$, $C_{(4)}$ and $C_{(4')}$ (6·65 μc) and similar to that of the carboxyl-carbon atom (5·2 μc). The latter value appears to be somewhat low, owing possibly to accidental dilution of the CO_2 from the carboxyl-carbon atom by a little extraneous CO_2. A similar partial degradation of the mycelial valine and of the valine added to the fermentation shows that the carboxyl-carbon atom of valine contains in each case about 20% of the total radioactivity.

Table 2. *Comparison of* ^{14}C *distribution in valine and penicillamine*

Uniformly ^{14}C-labelled L-valine was added to a fermentation of *P. chrysogenum*, WIS 51–20 (Expt. 8, Table 1). Figures in parentheses give the distribution of ^{14}C in the degradation products as a percentage of the initial radioactivity before degradation.

		Radioactivity in degradation products (μc/mole)	
Compound degraded	Radioactivity (μc/mole)	*iso*Butyraldehyde 2:4-dinitrophenylhydrazone ($C_{(2)}$–$C_{(4')}$)	$BaCO_3$ ($C_{(1)}$)
Valine added	1830	1500 (81)	385 (21)
Valine isolated from mycelial protein	63·2*	52·7 (83)	13·4 (21)
Penicillamine obtained by degradation of penicillin	32·5*	26·6 (82)	5·2 (16)

* After dilution with carrier.

Table 3. *Conversion of labelled valine and β-hydroxyvaline into penicillin and mycelial protein valine*

Penicillin was isolated as the *N*-ethylpiperidine salt, which was analysed for ^{15}N. The reported ^{15}N content has therefore been corrected for dilution by one atom of non-isotopic nitrogen by multiplying the observed values by 1·5. For experimental details and isotope content of the labelled amino acids added to the fermentations see Table 1 and text.
– Signifies value not determined.

			Isotope content of isolated compound		Dilution of added isotope	
Expt.	Labelled amino acid added	Compound isolated	^{14}C (μc/mole)	^{15}N (atom % excess)	^{14}C	^{15}N
1	DL-[α-^{14}C:^{15}N]Valine	Penicillin	28·9	0·120	7·7	39
		Mycelial valine	23·7	0·119	9·4	39
2		Penicillin	75·8	–	6·7	–
3		Penicillin	84·1	–	4·9	–
4		Penicillin	37·2	0·081	9·6	56
		Mycelial valine	25·4	0·077	14	58
5		Penicillin	37·1	–	9·1	–
6		Penicillin	9·5	–	10·5	–
8	L-[U-^{14}C]Valine + DL-[^{15}N]valine*	Penicillin	260	0·404†	7·0	38·5
		Mycelial valine	199·5	0·464	9·2	33·5
10	DL-β-Hydroxy[*carboxy*-^{14}C]valine	Penicillin	5·71‡	–	35	–
		Mycelial valine	1·14	–	173	–

* See footnote to Table 1.

† Calculated from the ^{15}N analysis of penicillin *N*-ethylpiperidine salt which had been diluted with a known amount of carrier.

‡ In a second experiment with DL-β-hydroxy[*carboxy*-^{14}C]valine (Expt. 9, Table 1) the radioactivity of the isolated penicillin was also very low (about 2 μc/mole) but was not measured accurately.

In these experiments, mixtures of DL-[^{15}N]valine and either DL-[α-^{14}C]valine or L-[U-^{14}C]valine were used as precursors (see Table 1), and the ^{15}N and ^{14}C contents of penicillin and valine from the mycelial protein are given in Table 3. The dilutions of ^{14}C for the biosynthesis of penicillin and mycelial protein were approximately 8 (range 4·9–10·5) and 11 (range 9·2–14) respectively. The rather wide range is probably due to differences in the times of addition of labelled valine to the fermentation and to variation in penicillin production (see Table 1). In all experiments, however, isotopic nitrogen was incorporated into penicillin and mycelial valine only with much greater dilution than was isotopic carbon, the average dilution being 45 and 50 respectively.

Degradation of the penicillin showed that both the penicillamine nitrogen atom and the side-chain nitrogen atom contained ^{15}N (Table 4). In three cases (I–III) the ^{15}N content of the penicillamine was not significantly above that of the average for both nitrogen atoms of penicillin, but in one case (IV) it was twice that of the side-chain nitrogen atom. It is noteworthy that in this experiment the isotope content of the mycelial valine was exceptionally high (see Table 3, Expt. 8), which may

Table 4. *Distribution of isotopic nitrogen in penicillin derived from* DL-[^{15}N]*valine*

For the degradation of penicillin, samples of penicillin *N*-ethylpiperidine salt from the following experiments were combined with carrier when necessary: I—Expts. 2, 3, 5 and 6; II—Expts. 1 and 7; III—Expt. 4; IV—Expt. 8. The ^{15}N content of the penicillin is the *average* value for both nitrogen atoms. All ^{15}N analyses have been corrected for non-isotopic nitrogen present in the derivatives analysed. – Signifies value not determined.

Substance	^{15}N Content (atom % excess) I	II	III	IV
Penicillin (average of both N atoms calc. from analysis of *N*-ethylpiperidine salt)	0·054±0·005	0·047±0·005	0·081±0·005	0·155±0·005
Penicillamine (isolated as *iso*propylidene derivative)	0·044±0·003 0·047±0·003	0·046±0·003 0·048±0·003	0·092±0·003	0·203±0·003
Penilloaldehyde (calc. from analysis of 2:4-dinitrophenylhydrazone)	–	–	–	0·105±0·015
Penilloaldehyde (calc. from analysis of *p*-bromophenylhydrazone)	–	–	–	0·078±0·009 0·102±0·009

Table 5. *Comparison of* ^{15}N *content of valine and some other amino acids isolated from mycelial protein*

The results have been calculated on the basis of 100 atom % excess ^{15}N in the added valine in order to compare the different experiments. – Signifies value not determined.

Amino acid isolated	^{15}N Content (atom % excess) Expt. 1	Expt. 4	Expt. 8
Alanine	1·66	1·45	1·99
Aspartic acid	1·53	–	–
Glutamic acid	1·66	1·29	–
Glycine	1·47	1·45	1·67
Valine	2·53	1·72	2·99

Table 6. *Relative incorporation of* ^{14}C *and* ^{15}N *from labelled valine into penicillin and mycelial protein*

The experimental results have been taken from Tables 1, 2, 4 and 7. In Expt. 4 DL-[α-^{14}C:^{15}N]valine was added to a fermentation, in Expt. 8 a mixture of L-[U-^{14}C]- and DL-[^{15}N]valine was used. The ^{14}C content is expressed as μc/mole, the ^{15}N content as atom % excess. The figures in parentheses in columns 6 and 7 give the change in the ^{14}C/^{15}N isotope ratio, obtained by dividing the ^{14}C/^{15}N ratio of the isolated valine or penicillamine by that of the valine added to the fermentation. Unless otherwise indicated, ^{15}N analyses are accurate to within ±0·003 and the standard error of the ^{14}C assays is about ±5%. These limits have been used to estimate the accuracy of the isotope ratios given in columns 6 and 7.

Compound	Isotope content Expt. 4 ^{14}C	^{15}N	Expt. 8 ^{14}C	^{15}N	^{14}C/^{15}N isotope ratio Expt. 4	Expt. 8
Added valine	355	4·50	1830	15·522	79±4	118±6
Mycelial valine	25	0·077	199·5	0·464	330±31 (4·2±0·6)	430±24 (3·65±0·4)
Penicillamine	38·2	0·092	246*	0·528*	415±37 (5·3±0·7)	465±28 (3·9±0·4)

* Calc. from isotope content of material diluted with carrier; ^{15}N analysis accurate to ±0·0075.

explain the relatively high incorporation of ^{15}N into the penicillamine nitrogen atom.

These results indicate that valine is reversibly deaminated before its incorporation into penicillin and mycelial protein. The deamination of valine is also shown by the extensive incorporation of isotopic nitrogen from [^{15}N]valine into other amino acids (Table 5). Thus alanine, glycine, glutamic acid and aspartic acid isolated from the mycelial protein of fermentations to which DL-[^{15}N]valine had been added contained relatively large amounts of ^{15}N. In one experiment (Expt. 4) the ^{15}N content of mycelial glycine and alanine was only 16 % less than that of valine.

A comparison of DL- and L-valine as penicillin precursors is given in Table 6. The incorporation of ^{14}C and ^{15}N into the penicillamine moiety of penicillin closely parallels that into mycelial valine in both experiments. Since in one experiment (Expt. 8) only the L-form of the added valine was labelled with ^{14}C, any preferential utilization of D-valine would have resulted in a marked decrease in the ^{14}C:^{15}N isotope ratio of the penicillamine moiety compared with that of mycelial valine. In each experiment, the ^{14}C:^{15}N ratio of mycelial valine is, however, identical with that of penicillamine within the limits of accuracy of the isotope analyses, indicating that D-valine is not utilized preferentially.

Penicillin biosynthesis from ^{14}C-labelled L- and D-valine by washed mycelium

The radioactivity of penicillin synthesized by washed mycelium in the presence of L- or D-[*carboxy*-^{14}C]valine is shown in Table 7. When the labelled valine was added at a final concentration of 0·005 %, L-[*carboxy*-^{14}C]valine gave rise to penicillin of higher radioactivity than that obtained from D-[*carboxy*-^{14}C]valine under identical conditions, although there was no significant difference in the rate of penicillin biosynthesis. The greatest difference in the conversion of L- and D-valine into penicillin was observed with short (1 or 2 hr.) incubation periods. Increase of the concentration of labelled valine to 0·02 % (Expt. WM 3) resulted in almost equal utilization of L- and D-valine in 4 hr.

Table 7 also gives the radioactivity in the CO_2 produced during incubation of labelled valine and that left in the mycelium after 1, 2 and 4 hr. The conversion of L-valine into CO_2 is much greater than that of the D-isomer. Metabolism of L-valine to CO_2 is, however, complete within 2 hr., since the radioactivities of CO_2 after 2 and 4 hr. are similar. On the other hand, metabolism of D-valine to CO_2 is very slow at first but increases between 2 and 4 hr.

In both experiments with 0·005 % L-[*carboxy*-^{14}C]-valine more radioactivity is present in the mycelium after 1 hr. than after 2 or 4 hr. With D-[*carboxy*-^{14}C]-

Table 7. *Incorporation of* L- *and* D-[carboxy-^{14}C]*valine into penicillin, mycelium and carbon dioxide by washed mycelium of* P. chrysogenum

All results are calculated for 100 ml. of medium (10 g. of wet mycelium). The specific radioactivity of the L- and D-[*carboxy*-^{14}C]valine was 3·32 μc/m-mole. Freshly harvested mycelium of *P. chrysogenum* WIS 51–20 was used for experiments WM 1 and WM 3. The mycelium used in experiment WM 2 had been stored for 1 day under O_2 at 0°.

Expt.	Labelled valine added (%)	Time of incubation (hr.)	Radioactivity in Mycelium (μmc)	Radioactivity in CO_2 (μmc)	Yield of penicillin (i.u./ml.)	Specific radioactivity of penicillin (μc/m-mole)
			L-[carboxy-^{14}C]*valine added*			
WM 1	0·005	1	42	15	4·2	1·52
		2	25	24	7·2	1·50
		4	31	26	10·1	1·16
WM 2	0·005	1	38	14	3·6	1·56
		2	19	30	6·3	1·14
		4	24	26	10·6	0·92
WM 3	0·02	4	157	98	7·2	1·27
			D-[carboxy-^{14}C]*valine added*			
WM 1	0·005	1	8	1·1	3·7	0·438
		2	11	4·8	6·9	0·634
		4	20	12·0	11·3	0·708
WM 2	0·005	1	8	1·1	3·4	0·398*
		2	12	2·0	6·2	0·439
		4	22	11·0	10·4	0·632
WM 3	0·02	4	175	52·0	7·6	1·25

* Sample not recrystallized.

valine the radioactivity of the mycelium increases throughout the incubation period. The difference in radioactivity incorporated into the mycelium from L- or D-[*carboxy*-^{14}C]valine was greatest after incubation for 1 hr. and decreased with time and thus paralleled that incorporated into penicillin.

With both L- and D-valine, a fourfold increase in concentration resulted in an approximately similar increase in their conversion into CO_2 at 4 hr., but the amount of radioactivity left in the mycelium was increased more than fourfold. No adequate explanation can at present be given for this finding.

Uptake of L- *and* D-*valine by mycelial cells*

The uptake by washed mycelium of L-valine, added to the incubation medium at a concentration of either 0·005 or 0·02 %, was essentially complete after 1 hr., but D-valine was absorbed only slowly, 40 % or more being left in the extracellular medium after 4 hr. (Fig. 1). The initial rate of uptake of L-valine appears to be at least 0·1 mg./min./g. of wet mycelium, whereas that of D-valine is only 0·01 mg./min./g. of mycelium.

In Fig. 2, the results given in Table 7 for the conversion of D-valine into penicillin have been calculated as a percentage of the conversion of L-valine into penicillin and compared with the uptake of D-valine, also expressed as a percentage of that of L-valine. It is clear that the less efficient utilization of D- compared with L-valine for penicillin biosynthesis is quantitatively accounted for by the slower uptake of the D-stereoisomer.

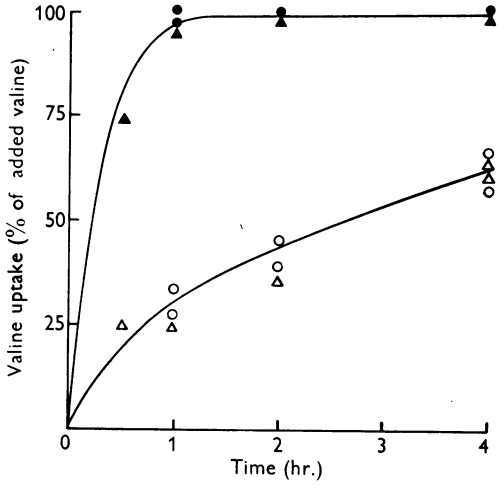


Fig. 1. Uptake of L- and D-valine by washed mycelium of *P. chrysogenum*, WIS 51–20. L- or D-[*carboxy*-^{14}C]Valine was added to washed mycelium at the following initial concentrations: ●, 0·005 % L-valine; ○, 0·005 % D-valine; ▲, 0·02 % L-valine; △, 0·02 % D-valine.

Comparison of valine and β-hydroxyvaline as penicillin precursors

Labelled β-hydroxyvaline was incorporated into penicillin only with a much greater dilution of isotope than valine (Table 3). Degradation of the penicillin derived from DL-β-hydroxy[*carboxy*-^{14}C]-valine shows that most of the isotope is located in the β-lactam carboxyl-carbon atom ($C_{(7)}$). The penicillamine moiety contained only 16 % of the total radioactivity, whereas ^{14}C-labelled valine is converted into penicillin containing 94–100 % of the total radioactivity in this portion of the molecule (Table 8). It is clear, therefore, that β-hydroxyvaline is not an intermediate in the utilization of valine for penicillin biosynthesis.

A comparison of the radioactivity of penicillin with that of several amino acids isolated from the mycelial protein of fermentations with labelled valine and β-hydroxyvaline (Table 9) also shows that hydroxyvaline is metabolized quite differently from valine. In the three experiments with labelled valine, the radioactivity of the mycelial valine was about 70 % of that of the penicillin and about 30 times that of any of the other mycelial amino

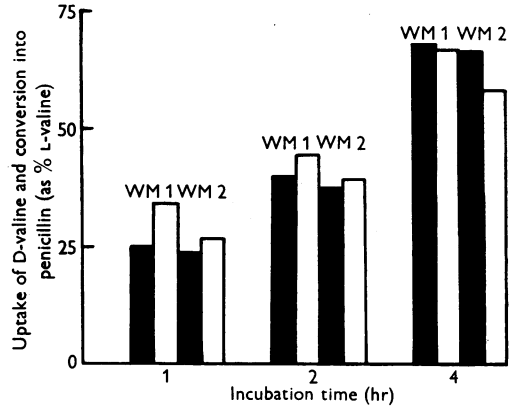


Fig. 2. Comparison of valine uptake and conversion into penicillin by washed mycelium of *P. chrysogenum*, WIS 51–20. The conversion of D-[*carboxy*-^{14}C]valine into penicillin relative to that of L-[*carboxy*-^{14}C]valine has been calculated from the results given in Table 7, columns 6 and 7. It is given by 100 × specific radioactivity × yield of penicillin synthesized in the presence of labelled D-valine divided by the specific radioactivity × yield of penicillin in the corresponding experiment with L-valine. The amount of valine remaining in the medium after incubation of the washed mycelium was estimated from the radioactivity of the valine isolated after addition of carrier (see Experimental section). Valine uptake was obtained by subtracting this figure from the amount of valine added initially. ■, Penicillin biosynthesis from D-valine as % from L-valine; □, uptake of D-valine as % of L-valine.

Table 8. *Distribution of* ^{14}C *in penicillin from fermentations containing* ^{14}C*-labelled valine or hydroxyvaline*

Penicillin was counted as the *N*-ethylpiperidine salt, penicillamine as the *iso*propylidene derivative, penilloaldehyde either as the 2:4-dinitrophenylhydrazone or the *p*-bromophenylhydrazone and the carboxyl carbon of the β-lactam ring ($C_{(7)}$) as $BaCO_3$. For other experimental details see text. When necessary the penicillin was diluted with carrier before degradation. The degradation of penicillin from Expt. 8 was carried out in duplicate. – Signifies value not determined.

Expt.	Radioactive amino acid tested as precursor	Radioactivity (μc/mole) in penicillin and degradation products: Penicillin (*A*)	Penicillamine (*B*)	Penilloaldehyde	$C_{(7)}$	Radioactivity in penicillamine (%) (100*B*/*A*)
2, 3, 5, 6	DL-[α-^{14}C]Valine	16·1	16·1	–	Inactive	100
1, 7		6·2	6·0	Inactive	Inactive	97·5
4		37·2	38·2	–	Inactive	103
8	L-[U-^{14}C]Valine	94·6*	90·9	0·6	4·3	95·5
			89·0	1·2	–	94·0
10	DL-β-Hydroxy[*carboxy*-^{14}C]valine	1·9*	0·3	Inactive	1·6	15·8

* After dilution with carrier.

Table 9. *Conversion of valine and β-hydroxyvaline into penicillin and amino acids by fermentations of* P. chrysogenum

In order to compare different experiments the radioactivity of the isolated compounds has been calculated for a specific radioactivity of the added amino acid (1 mc/mole). The amino acids were isolated from the mycelial protein. – Signifies value not determined.

Compound isolated	Radioactivity (μc/mole): Valine expt. (1)	Valine expt. (4)	Valine expt. (8)	Hydroxyvaline expt. (10)
Alanine	0·40	0·20	3·32	1·1
Aspartic acid	0·54	–	–	0·48
Glutamic acid	1·08	1·27	–	Inactive
Glycine	0·29	0·24	1·02	30·4
Valine	106	71·5	109	5·7
Penicillin	130	105	142	29

acids isolated. With labelled hydroxyvaline, however, the highest radioactivity was found in the mycelial glycine. Penicillin, labelled largely in the β-lactam portion as already mentioned, had a radioactivity similar to that of the mycelial glycine. The radioactivity of the mycelial valine was only about 20% of that of the glycine and thus corresponded approximately to that of the penicillamine moiety of penicillin (Table 8).

DISCUSSION

The present experiments with [α-^{14}C]valine and uniformly ^{14}C-labelled valine confirm our earlier results with DL-[γγ′-^{14}C]valine (Arnstein & Grant, 1954*a*), which indicated that valine is used by *Penicillium chrysogenum* as a precursor of the penicillamine moiety of penicillin (I). Utilization of the intact carbon chain of valine has now been demonstrated by the equal incorporation of uniformly labelled valine into the carboxyl carbon atom and $C_{(2)}$–$C_{(4')}$ of penicillamine. This conclusion is in agreement with the work of Stevens, Vohra & De Long (1954), who showed that DL-[*carboxy*-^{14}C]-valine gives rise to penicillin labelled in the carboxyl-carbon atom of penicillamine.

The low ^{15}N content of both penicillin and mycelial valine isolated from fermentations to which DL-[^{15}N]valine had been added indicates that valine is oxidatively deaminated or transaminated, presumably to dimethylpyruvic acid (α-oxo*iso*-valeric acid), at a rate which must be considerably faster than penicillin or protein synthesis. *Penicillium chrysogenum* contains both an L- and a D-amino acid oxidase (Knight, 1948; Emerson, Puziss & Knight, 1950), and dimethylpyruvic acid has been isolated from a related organism, *Aspergillus niger* (Ramachandran & Walker, 1951). It is noteworthy, however, that *A. niger* is apparently unable to synthesize dimethylpyruvate from valine.

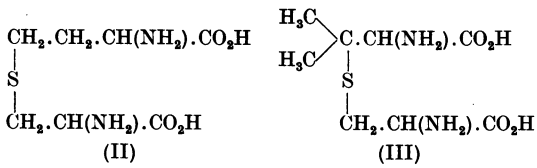
The relatively efficient incorporation of carbon-labelled valine into the mycelial protein implies the re-utilization of the carbon chain by amination of the keto acid to L-valine. The equally efficient utilization of carbon-labelled valine for penicillin biosynthesis does not, however, require the participation of D-valine as an obligatory intermediate. Although the relevant carbon atom of penicillin [$C_{(3)}$, formula (I)] has the D-configuration, it is conceivable that L-valine reacts with L-cysteine to give an intermediate which might subsequently be converted into penicillin by reactions involving the introduction of the D-configuration. Thus the following would be a possible biosynthetic pathway:

L-valine → L-cysteinyl-L-valine →
L-cysteinyl-D-valine → penicillin.

On the other hand, it should be pointed out that D-valine has not been excluded as a possible intermediate, since the preferential utilization of L-valine for penicillin synthesis by washed mycelium

is due to its faster uptake by the cells compared with that of D-valine. When allowance is made for the differences in uptake, both L- and D-valine appear to be converted into penicillin to an equal extent. Recently it has been reported that growing cultures of *P. chrysogenum* utilize D-valine for penicillin biosynthesis only after an initial lag period, whereas L-valine is used immediately (Stevens, Inamine & De Long, 1956). It was suggested that D-valine is therefore not a normal intermediate in penicillin synthesis, but possible differences in the uptake of the D- and L-amino acid by the cells were apparently not considered. In view of our present results it seems likely that this observed lag period in the utilization of D-valine must also be ascribed to its slower uptake.

In an attempt to elucidate the mechanism of penicillin biosynthesis from valine in more detail, β-hydroxyvaline was tested as a possible intermediate. By analogy with the formation of cystathionine (II) from cysteine by *Neurospora* (Horowitz, 1947), the thio ether link of penicillin might have been expected to be synthesized by condensation of cysteine with hydroxyvaline, as has been suggested by Hockenhull, Ramachandran & Walker (1949), possibly with the intermediary formation of dimethyllanthionine (III). Such a



(II) (III)

mechanism would require the incorporation of labelled hydroxyvaline into the penicillamine moiety with less dilution of isotope than that found with valine. Our experiments show, however, that DL-β-hydroxy[*carboxy*-^{14}C]valine is utilized much less efficiently than valine for penicillin biosynthesis. Moreover, the isotope was incorporated mainly into $C_{(7)}$ of penicillin (I). These results and the high ^{14}C content of glycine isolated from the mycelial protein suggest that hydroxyvaline is converted into penicillin only after breakdown to glycine and, presumably, acetone. Such a cleavage of the αβ carbon–carbon bond of hydroxyvaline has been demonstrated in animal tissue (Vilenkina, 1949). Glycine is known to be an indirect precursor of the β-lactam ring of penicillin (Arnstein & Grant, 1954*a*), probably by the metabolic pathway glycine → serine → cysteine, and this mechanism would account for the labelling of $C_{(7)}$ of penicillin in the hydroxyvaline experiment.

Although β-hydroxyvaline itself has thus been excluded as a penicillin precursor a peptide such as cysteinyl-β-hydroxyvaline could still be an intermediate in penicillin biosynthesis, if it were synthesized by oxidation of cysteinylvaline rather than by reaction of cysteine and hydroxyvaline. Although this possibility may seem unlikely, several more obvious mechanisms have now been excluded. Thus ββ-dimethyllanthionine is apparently not a penicillin precursor (Stevens, Vohra, Moore & De Long, 1954) and phenylacetylcysteine, which could give rise to phenylacetyldimethyllanthionine and thence penicillin without the intermediary formation of free dimethyllanthionine, is not utilized as such for penicillin biosynthesis (Arnstein, Clubb & Grant, 1954). The initial step of penicillin biosynthesis therefore probably involves the condensation of cysteine and valine by a peptide link, whereas the thio ether may be introduced subsequently by the intramolecular cyclization of an intermediate such as cysteinyl-β-hydroxyvaline or cysteinyl-αβ-dehydrovaline.

SUMMARY

1. Uniformly ^{14}C-labelled L-valine, DL-[α-^{14}C]-valine, DL-[^{15}N]valine and DL-β-hydroxy[*carboxy*-^{14}C]valine have been compared as penicillin precursors in fermentations of two strains of *Penicillium chrysogenum*. The utilization of valine for penicillin biosynthesis has also been studied with suspensions of washed mycelium to which L- or D-[*carboxy*-^{14}C]-valine was added.

2. Degradation of penicillin derived from uniformly ^{14}C-labelled valine shows that the carbon chain of the amino acid is used intact for biosynthesis of the penicillamine moiety of penicillin.

3. Valine labelled in the carbon chain with ^{14}C was incorporated into penicillin with much less dilution of isotope than was ^{15}N-labelled valine, indicating the rapid deamination of valine by a reversible reaction such as either an oxidative deamination or a transamination to dimethylpyruvic acid.

4. The conversion of L-valine into penicillin by washed mycelium is more efficient than that of D-valine. This difference appears to be due to the slower uptake of D-valine by the cells rather than to the preferential utilization of the L-enantiomorph for penicillin biosynthesis.

5. Experiments with β-hydroxy[*carboxy*-^{14}C]-valine show that this amino acid is not an intermediate in the biosynthesis of penicillin from valine. The distribution of isotope in penicillin and in certain amino acids of the mycelial protein suggests that hydroxyvaline is metabolized to glycine and, presumably, acetone.

6. The synthesis of DL-[^{15}N]valine and DL-β-hydroxy[*carboxy*-^{14}C]valine is described.

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Glycerylphosphorylcholine and Phosphorylcholine in Semen, and their Relation to Choline

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Mammalian seminal plasma represents one of the richest natural sources of free choline and water-soluble choline compounds (Mann, 1954). However, as shown by Kahane & Lévy (1936, 1937), free choline accumulates in human seminal plasma only after ejaculation, as the result of a hydrolytic process which takes place in voided semen, on standing. Investigations on the nature of the choline precursor, which were carried out independently in the laboratories of Kahane in France (Kahane & Lévy, 1938, 1945, 1949; Diament, Kahane & Lévy, 1952, 1953; Diament, 1954, 1955) and of Lundquist in Denmark (Lundquist, 1946, 1947*a*, *b*, 1953), led to somewhat conflicting results, particularly as regards (i) the ratio between free choline and the simultaneously liberated inorganic phosphate, and (ii) the nature of the phosphorylated choline precursor. It is the purpose of this paper to report the results of an investigation of the nature and distribution of choline compounds in the semen and male accessory secretions of various species. As well as human semen, the study included material from monkey, ram, goat, bull, boar, stallion, rat, rabbit, hedgehog and cock.

EXPERIMENTAL

Collection of semen and of secretions from male accessory organs. Human semen from donors at the Fertility Clinic was kindly made available by Dr H. A. Davidson. The semen of ram, bull, boar, goat, rabbit and stallion was