sulphatases *A* and *B* were determined by paper electrophoresis. There were considerable variations in this ratio and sulphatase *A* appeared to be lacking in the lower vertebrates.

6. Sulphatases *A* and *B* were localized predominantly in the mitochondrial and microsomal fractions. Sulphatase *C* occurred only in the microsomes.

7. Although the distribution of sulphatases *A* and *B* was consistent with them occurring in the lysosomes they could not be completely extracted from the mitochondrial and microsomal fractions with water, so that it is probable that not all of these enzymes are localized in the lysosomes.

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The Biosynthesis of Penicillin

8. INVESTIGATION OF CYCLIC CYSTEINYLVALINE PEPTIDES AS PRECURSORS*

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The demonstration of the intact incorporation of L-cystine, presumably after reduction to cysteine (Arnstein & Grant, 1954a, b; Arnstein & Crawhall, 1957) and of the utilization of the carbon chain of

valine for the biosynthesis of the β -lactam-thiazolidine ring system of penicillin (I) (Arnstein & Grant, 1954a; Stevens, Vohra & De Long, 1954; Arnstein & Clubb, 1957) has led to speculation on the mechanism by which these amino acids combine, and the nature of the more immediate precursors of the penicillin molecule so formed.

Although the utilization of valine nitrogen by

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the mould could not be demonstrated conclusively owing to the rapid transamination which it undergoes on addition to the fermentation medium (Arnstein & Clubb, 1957), D-valine has now been excluded as a penicillin precursor (Arnstein & Margreiter, 1958). It is probable therefore that the penicillamine moiety of penicillin is derived from either L-valine or the corresponding keto acid, α -oxoisovaleric acid. Several metabolic pathways by which these compounds might combine with cysteine may be postulated, and of these some of the more obvious have already been excluded. For example, the formation of $\beta\beta$ -dimethyl-lanthionine (II) in a manner analogous to that of cystathionine in *Neurospora* (Horowitz, 1947), that is by condensation of cystine with β -hydroxyvaline, does not appear to take place (Arnstein & Clubb, 1957; Stevens, Vohra, Moore & De Long, 1954).

(R = C₆H₅·CH₂ in benzylpenicillin)

Phenylacetylcysteine, from which phenylacetyl-dimethyl-lanthionine (VIII) could be formed without involving dimethyl-lanthionine (II) itself, has also been excluded (Arnstein, Clubb & Grant, 1954). It therefore seemed likely that a peptide bond is first formed between cystine (or cysteine) and valine and that the thio ether linkage is introduced subsequently by the intramolecular cyclization of an intermediate such as cysteinyl- β -hydroxyvaline or cysteinyl- $\alpha\beta$ -dehydrovaline. A reaction of this kind would give rise to a cyclic derivative (VI) of cysteinylvaline containing both thio ether and peptide bonds in a seven-membered ring.

The present paper describes the synthesis of peptides of this type, and preliminary experiments designed to test whether they are involved as intermediates in penicillin biosynthesis. Since the synthesis of these compounds was lengthy and the overall yields were low, an isotopic-competition

method was used, ¹⁴C-labelled valine being added alone or together with the peptides to a suspension of mycelial cells synthesizing the antibiotic. A reduction in the specific radioactivity of penicillin synthesized in the presence of the unlabelled peptides would indicate that they are more immediate precursors than valine itself. Contrary to expectation, no indication of the utilization of these compounds for penicillin biosynthesis was obtained and further investigations with labelled material were therefore not undertaken. Two substituted thio ether compounds related to $\beta\beta$ -dimethyl-lanthionine, namely S-(2-acetamido-2-carboxyethyl)-DL-penicillamine (VII) and S-(2-phenylacetamido-2-carboxyethyl)-DL-penicillamine (VIII), resulting from the acid hydrolysis of (III) and (IV) respectively, were also included in the tests but proved equally ineffective in reducing the incorporation of labelled valine into penicillin.

EXPERIMENTAL

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

Method for incubation of washed mycelial cells. Washed mycelium of *Penicillium 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 incubation medium contained 0.005% of potassium phenylacetate, 0.25% of glucose, and 0.005% of L-[carboxy-¹⁴C]valine (0.0289 μ c/mg.) and the suspension of mycelium was incubated in duplicate conical flasks (250 ml. for 40 ml. of medium) on a rotary shaker (250 rev./min.) at 24° for 4 hr. The compounds to be tested were added at a final concentration of 0.025%. The penicillin concentration of the medium was determined by cup-plate bioassay with *Bacillus subtilis* (I.C.I. strain NCTC 8241) as test organism (Humphrey & Lightbown, 1952) at the end of the experiment, and the rate of synthesis under these conditions was about 4 i.u./ml./hr.

Isolation and purification of penicillin. Inactive carrier penicillin (40 mg. of Glaxo sodium benzylpenicillin, potency 1650 i.u./mg.) was added to the incubation broth after removal of mycelium by filtration and sampling for bioassay. The solution was cooled to 4° and acidified with 25% (v/v) phosphoric acid to pH 2, and the penicillin extracted with ether. The volume of the solution was reduced by successive partitioning of penicillin between ether at pH 2 and phosphate buffer at pH 7.0 (Smith & Hockenhull, 1952). Finally, the penicillin was isolated as the N-ethylpiperidine salt (28-30 mg.) which was purified to constant radioactivity by recrystallization from chloroform-acetone (1:4, v/v).

Chemical preparations

L-[carboxy-¹⁴C]Valine. This material was obtained by resolution of DL-[carboxy-¹⁴C]valine supplied by the Radio-

Fig. 1. Synthesis of cyclic peptides related to penicillin. (R = CH₃·CO, C₆H₅·CH₂·CO or C₆H₅·CH₂·O·CO).

Table 1. *R_F* values of peptides and intermediates

All paper chromatograms were run on Whatman no. 1 paper. Solvent systems had the following composition (by vol.): A, Butanol-acetic acid-water (63:10:27); B, pentan-1-ol-pyridine-water (7:7:6); C, phenol-water (5:2) (NH₃ present in the chromatography tank).

All substances were detected by spraying the paper with 0.1% ninhydrin in butanol containing 2% of water, unless otherwise stated. For chemical formulae of the substances see text and Fig. 1. — Signifies value not determined.

Substance	Solvent systems		
	A	B	C
(XII; R = CH ₃ ·CO)	0.38	0.32	—
(XIII; R = CH ₃ ·CO)	—	0.48	0.85
(III)	0.69*	0.25*	—
(XII; R = C ₆ H ₅ ·CH ₂ ·CO)	0.66	0.35	—
(XIII; R = C ₆ H ₅ ·CH ₂ ·CO)	0.61	0.53	—
(IV)	0.78–0.84†	—	—
(XII; R = C ₆ H ₅ ·CH ₂ ·O·CO)	0.63–0.66	0.38	—
(XIII; R = C ₆ H ₅ ·CH ₂ ·O·CO)	0.69	—	—
(V)	0.82–0.88*†	—	—
(VI)	0.28–0.33	0.25	0.78
(VII)	0.16	0.23	0.53
(VIII)	0.33–0.43	0.06	0.66

* Material detected by chlorine-starch-iodide technique (Rydon & Smith, 1952) and also with bromophenol blue and methyl red indicators. Repeated attempts to detect the cyclic phenylacetylpeptide (IV) by these methods were, however, unsuccessful.

† Material detected by spraying with 1% AgNO₃ in aq. N-NH₃, drying and heating the paper at 115°, when light spots on a brown background were obtained. Alternatively BDH Universal Indicator (British Drug Houses Ltd.) could be used, but the spots were more difficult to detect.

chemical Centre, Amersham, by means of the brucine salts of *N*-formyl-DL-valine (Arnstein & Clubb, 1957).

Synthesis of peptides. The method used for synthesizing the cyclic peptides (III)–(VI) is outlined in Fig. 1. All melting points were measured on a micro-block apparatus and are uncorrected. The *R_F* values of peptides and intermediates are given in Table 1.

6-Acetamido-2:2-dimethyl-5-oxohexahydro-1:4-thiazepine-3-carboxylic acid (III). Freshly distilled pyruvic acid (44 g., 0.5 mole) was condensed with acetamide (59 g., 1.0 mole, twice recrystallized from acetone) by azeotropic distillation in toluene (150 ml.) at a bath temperature of 160°. After 3 hr. 90% of the theoretical volume of water

had been collected. The toluene was decanted and the brown crystalline residue washed with warm ethanol, yielding diacetamidopropionic acid (IX; R = CH₃·CO) (46.5 g., 50%), m.p. 197°.

The acid (IX; 15 g.) was partially hydrolysed in acetic acid containing 1 drop of conc. HCl/100 ml. (cf. Bergmann & Grafe, 1930). Approximately half the acetic acid was removed by evaporation *in vacuo*, and on cooling acetamidoacrylic acid (X; R = CH₃·CO) crystallized in fine needles. The product was washed with ice-cold water (3 × 10 ml.) and dried, m.p. 205° (yield 7.8 g., 75%).

Acetamidoacrylic acid was esterified according to Rothstein (1949) and the methyl ester (XI; R = CH₃·CO) was obtained as almost colourless needles (yield 73%), m.p. 52–53° after crystallization from light petroleum (b.p. 40–60°).

The product (6 g., 42 m-moles) was dissolved in water (137 ml.) and added to a solution in water (80 ml.) of *iso*-propylidene-DL-penicillamine hydrochloride (4.7 g., 42 m-moles) which had been boiled for 3 min. to hydrolyse the thiazolidine and neutralized with aq. 1.5N-NH₃. The mixed solution was heated in a steam bath with a slow stream of H₂ passing through. After 3 hr. the solution gave a negative thiol reaction with alkaline sodium nitroprusside (cf. Süs, 1950). Inorganic salt was removed from the solution by the addition of 1% silver acetate (700 ml.). Hydrogen sulphide was passed through and AgCl and Ag₂S were removed simultaneously by centrifuging. The supernatant solution was evaporated to dryness and the brown residue was extracted continuously with hot ethanol. S-(2-Acetamido-2-methoxycarbonylthiethyl)penicillamine (XII; R = CH₃·CO) was obtained as a white microcrystalline solid on addition of an equal volume of ether to the ethanol extract (5.8 g., m.p. 175–176°; second crop 4.8 g., m.p. 166°; total yield 85%). A sample was recrystallized from water-ethanol, m.p. 175–176° (Found: C, 45.1; H, 7.1; N, 9.5; S, 11.0. C₁₁H₂₀O₅N₂S requires C, 45.2; H, 6.8; N, 9.6; S, 11.0%).

The ester (XII; 5.8 g., 20 m-moles) was suspended in cold ethanol (250 ml.) and 100% hydrazine hydrate (3.75 ml., 75 m-moles) was added. On warming, a clear solution was obtained and was kept at 37° for 60 hr. The ethanol was removed by evaporation *in vacuo* at a temperature not exceeding 50°. S-(2-Acetamido-2-hydrazinocarbonylthiethyl)penicillamine (XIII; R = CH₃·CO) was obtained as a white amorphous solid (2.3 g., 40%) by dissolving the residue in a small volume of water and adding ethanol to turbidity. The product was recrystallized from water-ethanol, m.p. 205–219° with decomposition (Found: C, 39.6; H, 7.0; N, 18.9; S, 10.6. C₁₀H₂₀O₄N₄S requires C, 41.1; H, 6.9; N, 19.2; S, 11.0%).

Cyclization of the hydrazide (XIII; $R=CH_3 \cdot CO$) was effected at high dilution in aqueous medium, pH 7.0 (cf. Sheehan & Richardson, 1954). The above product (2.57 g., 8.75 m-moles) was dissolved in 0.2N-HCl (87.5 ml., 17.5 m-moles) and cooled to 0°. Sodium nitrite (0.605 g., 8.75 m-moles) in water (4.4 ml.) was added and the mixture swirled gently at 0° for 15 min. It was then poured into ice-cold water (5 l.), $NaHCO_3$ (11 g.) was added, and the solution was stored at 4° for 40 hr. The pH was adjusted to 4.5 by the addition of 2N-HCl (approx. 60 ml.) and the solution was concentrated to 135 ml. *in vacuo* at <45°. This solution was extracted with butanol (3 × 50 ml.), the extract concentrated *in vacuo* (to 100 ml.) and inorganic salt, which separated as a fine white cryst. solid on storage at 4° overnight, was removed. The butanolic solution was evaporated to dryness and the residue was crystallized from acetone (m.p. 215°; yield 0.9 g., 40%). On slow crystallization from water, the *cyclic peptide* (III) was obtained as fine needles, m.p. 215–216° [Found: C, 45.9; H, 6.2; N, 10.6; S, 12.7%; mol.wt. (Rast) 310. $C_{10}H_{14}O_4N_2S$ requires C, 46.1; H, 6.2; N, 10.8; S, 12.3%; mol.wt. 260]. This material gave no colour with ninhydrin but was detected on paper chromatograms by the chlorine–starch–iodine technique (Rydon & Smith, 1952) and with suitable indicators (see Table 1).

2:2-Dimethyl-5-oxo-6-phenylacetamidoheptahydro-1:4-thiazepine-3-carboxylic acid (IV). This compound was prepared by a series of reactions analogous to those for the preparation of (III). Phenylacetamidacrylic acid, m.p. 180–183°, was prepared by the method of Süs (1950). The *methyl ester* (XI; $R=C_6H_5 \cdot CH_2 \cdot CO$) was obtained by refluxing the silver salt with methyl iodide for 3 hr. On crystallization from light petroleum (b.p. 60–80°) it formed white plates, m.p. 56° (yield 50%) (Found: C, 65.9; H, 5.9; N, 6.2; $C_{12}H_{13}O_3N$ requires C, 65.8; H, 6.0; N, 6.4%).

The ester (6.0 g., 29.5 m-moles) was dissolved in ethanol (100 ml.) and heated on a steam bath in a slow stream of H_2 for 3 hr. with a solution of isopropylidenepenicillamine hydrochloride (6.8 g., 30 m-moles) in water (100 ml.) which had been boiled for 3 min., cooled and neutralized with 1.5N- NH_3 . The reaction mixture was freed from inorganic salt and purified as before. The *product* (XII, $R=C_6H_5 \cdot CH_2 \cdot CO$) (5.9 g., 54%) was obtained as a white powder from ethanol-ether, m.p. 164° (Found: C, 54.6; H, 6.7; N, 7.5; S, 8.9. $C_{17}H_{24}O_5N_2S$ requires C, 55.4; H, 6.6; N, 7.6; S, 8.7%).

The ester (XII; $R=C_6H_5 \cdot CH_2 \cdot CO$) (5.6 g., 15 m-moles) was suspended in ethanol (400 ml.), and 100% hydrazine hydrate (3.0 ml.) was added. The mixture was kept at 37° for 16 hr. and then heated on a steam bath for a further 6 hr. Removal of the ethanol by evaporation *in vacuo* and crystallization of the residue from water-ethanol gave the *hydrazide* (XIII; $R=C_6H_5 \cdot CH_2 \cdot CO$) as a white crystalline powder (1.35 g., 24%), m.p. 219° with decomposition (Found: C, 52.0; H, 6.1; N, 15.1; S, 9.0. $C_{16}H_{24}O_4N_4S$ requires C, 52.2; H, 6.6; N, 15.2; S, 8.7%).

The *hydrazide* (0.81 g., 2.2 m-moles) was converted into the acid azide and cyclized at high dilution (1.5 l.) as described above for the acetyl compound. After purification as for (III), the *cyclic peptide* (IV) crystallized from a small volume of water in fine needles (0.2 g., 27%), m.p. 194–195° (Found: C, 56.9; H, 6.1; N, 8.5; S, 9.7%; mol.wt. (Rast) 347. $C_{16}H_{20}O_4N_4S$ requires C, 57.1; H, 6.0; N, 8.3; S, 9.5%; mol.wt. 336).

6-Amino-2:2-dimethyl-5-oxohexahydro-1:4-thiazepine-3-carboxylic acid (VI). Benzyl carbamate (50 g., 0.33 mole) was condensed with pyruvic acid (25 ml., 0.37 mole) by azeotropic distillation in benzene (250 ml.) for 2.5 hr. at a bath temp. of 110–115°. The residue, after the benzene had been decanted, crystallized on cooling and was recrystallized from ethyl acetate (yield, 15 g., 30%; m.p. 138–140°). Benzyl carbamate (approx. 20 g.) was recovered from the mother liquors. *α*-Dibenzoyloxycarbonamidopropionic acid (IX, $R=C_6H_5 \cdot CH_2 \cdot O \cdot CO$) (15 g., 41 m-moles) was partially hydrolysed by the method of Frankel & Riechmann (1952) to *α*-benzoyloxycarbonamidacrylic acid, which was recrystallized from ethanol–water (yield 8 g., 85%; m.p. 103–105°).

The product (8 g., 36 m-moles) was converted into the silver salt (yield, 98%) which was stirred with methyl iodide (70 ml., 150% excess) and a trace of quinol at room temp. for 2 hr. The mixture was filtered and the solid refluxed with a further portion of methyl iodide (30 ml.) for 1 hr. Methyl iodide was removed from the combined filtrates at atmospheric pressure. The *methyl ester* (XI, $R=C_6H_5 \cdot CH_2 \cdot O \cdot CO$) crystallized as dense needles when the residue was dissolved in light petroleum (b.p. 40–60°) and the solution cooled to –70°. It was recrystallized from the same solvent (yield 6.0 g.; 70%; m.p. 34–35°) (Found: C, 61.4; H, 5.7; N, 6.0. $C_{12}H_{13}O_3N$ requires C, 61.3; H, 5.6; N, 6.0%).

The *methyl ester* (5.88 g.; 25 m-moles) was condensed with penicillamine (25 m-moles) in ethanol–water (100 ml.: 50 ml.) containing a trace of quinol. The mixture was saturated with hydrogen and kept at 37° for 112 hr. After removal of inorganic salts as before, the *product* (XII, $R=C_6H_5 \cdot CH_2 \cdot O \cdot CO$) was twice recrystallized from methanol–ether (yield 3.8 g., 40%; m.p. 172°) (Found: C, 53.1; H, 6.3; N, 7.0; S, 8.6. $C_{17}H_{24}O_5N_2S$ requires C, 53.1; H, 6.3; N, 7.3; S, 8.3%).

A solution of this ester (2.7 g., 6.75 m-moles) in methanol (75 ml.) containing 100% hydrazine hydrate (1.35 ml.) was kept at 37° for 72 hr. The solution was decanted off some crystals which had formed on the flask wall (0.65 g.; m.p. 225° with decomposition) and refluxed gently for 20 min., when more white solid formed (0.31 g.; m.p. 228°). A third crop of the *hydrazide* (0.17 g.; m.p. 225°) was obtained when more hydrazine hydrate (0.5 ml.) was added to the mother liquors and refluxed for a further 30 min. The *hydrazide* (XIII, $R=C_6H_5 \cdot CH_2 \cdot O \cdot CO$) (total yield 1.13 g., 44%) was used without further purification but a sample was recrystallized from water-methanol (m.p. 225°) (Found: C, 48.8; H, 6.1; N, 14.5; S, 8.4. $C_{16}H_{24}O_4N_4S$ requires C, 50.0; H, 6.3; N, 14.6; S, 8.3%).

The *hydrazide* (0.96 g.; 2.5 m-moles) was converted into the acid azide and cyclized at high dilution (1.5 l.) as before. After being stored for 40 hr. at 4°, the solution was concentrated to 140 ml. *in vacuo*, cooled to 5°, and acidified to pH 4.5 with conc. HCl. After extraction with butanol (1 × 50, 2 × 25 ml.), the aqueous solution was neutralized to pH 7.0 and concentrated to 65 ml. A second butanol extraction (2 × 25 ml.) was made at pH 4.5, the combined extracts were dried over anhydrous Na_2SO_4 and lyophilized. The residue (0.7 g.) was thoroughly extracted with warm water (3 × 10 ml.) and the water-insoluble residue (0.6 g.) was crystallized from 50% aqueous acetone, ethanol or ethanol–water, yielding 0.34 g. (43%) of the *cyclic peptide* (V), m.p. 204° [Found: C, 54.4; H, 5.7;

N, 8.3; S, 8.7%; mol.wt. (Rast) 304; $C_{16}H_{20}O_5N_2S$ requires C, 54.5; H, 5.7; N, 8.0; S, 9.1%; mol.wt. 352].

The benzyloxycarbonyl group was removed by treating 100 mg. of the peptide (V) with *m*-HBr in acetic acid (5.0 ml.) (Ben-Ishai & Berger, 1952) for 2 hr. at room temp. in a flask fitted with a $CaCl_2$ tube. The mixture was warmed in a bath at 45–50° for a few minutes and lyophilized. The hydrobromide (68–72 mg., 80–85%) was washed with several portions of dry ether. The free base was obtained by dissolving the hydrobromide (50 mg.) in a little water, making alkaline with aq. NH_3 (sp.gr. 0.88) and evaporating the solution to dryness. The white crystalline powder was recrystallized from dil. aq. NH_3 -ethanol (yield, 22.4 mg., 61%; m.p. above 250° with charring). The product (VI) gave a characteristic yellow colour reaction with ninhydrin, changing to mauve, and was chromatographically pure (see Table 1) (Found: C, 43.7; H, 6.6; N, 12.8; S, 15.8. $C_8H_{14}O_5N_2S$ requires C, 44.1; H, 6.5; N, 12.8; S, 14.7%).

S-(2-Acetamido-2-carboxyethyl)penicillamine (VII). Acid hydrolysis of (III) (200 mg.) with 12*N*-HCl (10 ml.) at 37° for 4 hr. resulted in the formation of (VII), as shown by paper chromatography. The mixture was frozen, HCl was removed *in vacuo*, and the free base obtained by passing an aqueous solution of the product through a column (diameter 1.5 cm.) containing Zeo-Karb 215 (10 g. wet wt. in H^+ form) and eluting with 0.033*N*- NH_3 . By fractionation of the eluate (3.0 ml. fractions at the rate of 30/hr.) contaminating dimethyl-lanthionine (II) was removed. The product (VII) was crystallized from water-acetone and recrystallized from water-ethanol (yield 50.7 mg., 31%; m.p. 228°) (Found: C, 43.4; H, 6.4; N, 9.9; S, 10.9. $C_{16}H_{18}O_6N_2S$ requires C, 43.2; H, 6.5; N, 10.1; S, 11.5%). For com-

parison, samples (m.p. 220°) of (VII) were also prepared by saponification of the ester (XII, $R=CH_3\cdot CO$) and by condensation of acetamidoacrylic acid with penicillamine (cf. Süs, 1950) (Found: C, 43.1; H, 6.8; N, 9.8; S, 11.5%). All these samples had identical R_F values in three different solvents (Table 1).

S-(2-Phenylacetamido-2-carboxyethyl)penicillamine (VIII). A sample of (VIII) was prepared by condensation of phenylacetamidoacrylic acid with penicillamine and was recrystallized from water (m.p. 196–197°; cf. Süs, 1950) (Found: C, 54.0; H, 6.0; N, 7.8; S, 8.9. $C_{16}H_{22}O_5N_2S$ requires C, 54.2; H, 6.3; N, 7.9; S, 9.0%). This material was identified by paper chromatography (see Table 1) with the product of hydrolysis of (IV) with 12*N*-HCl at 37° for 4 hr.

$\beta\beta$ -Dimethyl-lanthionine (II). *S*-(2-Acetamido-2-carboxyethyl)penicillamine (VII, 1.46 g., 5 m-moles) was boiled with 20% HCl (50 ml.) for 5 hr. The solution was evaporated to dryness, and passed through a column of Zeo-Karb 215 resin (H^+ form). After the column had been washed free from acid, the product was eluted with ammonia and crystallized from water-ethanol (yield 0.7 g., 59%; m.p. 250° with decomposition).

RESULTS

The yield and radioactivity of penicillin, synthesized by washed mycelium of *P. chrysogenum* in the presence of L-[carboxy- ^{14}C]valine alone or together with the synthetic compounds whose precursor activity was to be tested, are shown in Table 2. The average production of penicillin was

Table 2. *Penicillin biosynthesis from L-[carboxy- ^{14}C]valine in the presence of certain peptides and thio ethers related to cystine and valine.*

The compounds tested were added at a concentration of 25 mg./100 ml. to a suspension of washed mycelium in the presence of L-[carboxy- ^{14}C]valine as described in the text. Penicillin production in control flasks was 12.5 i.u./ml. in Expts. nos. 1–4 and 10.5 i.u./ml. in Expts. nos. 5–7, the specific radioactivity of the penicillin *N*-ethylpiperidine salt being 2.54 and 2.23 $\mu C/g.$ in the two series of experiments respectively.

Expt. no.	Compound tested	Yield of penicillin (i.u./ml.)	Specific radioactivity of penicillin <i>N</i> -ethylpiperidine salt ($\mu C/g.$)	Mean specific radioactivity (as % of control)
1	Acetyl peptide (III)	11.5 12.7	2.52 2.74	103.5
2	Phenylacetyl peptide (IV)	11.9 12.1	2.78 2.74	108.7
3	Dimethyl-lanthionine	11.8 12.3	2.89 2.96	114.9
4	Acetyldimethyl-lanthionine (VII)	13.6 12.1	2.36 2.74	100.4
5	Phenylacetyldimethyl-lanthionine (VIII)	11.2 6.5*	1.90 2.95	85.2
6	Amino peptide (VI)†	8.8 9.0	2.31 2.49	107.6
7	Phenylacetyl-L-cysteinyl-D-valine‡	10.1 5.6*	2.50 3.46	112.1

* Low penicillin yield: flask rejected in calculation of column 5.

† Compound added as hydrobromide.

‡ Supplied by Merck and Co. and added at a final concentration of 0.005% only.

not affected by the addition of the test compounds, being 98 and 93 % (Expts. nos. 1-4 and 5-7 respectively) of the value in the corresponding control experiments. In Expt. no. 6 the hydrobromide of the peptide (VI) was used, but an equivalent amount of potassium hydroxide was added to maintain the neutral pH of the medium. In a further control experiment containing the same concentration of potassium bromide, the penicillin yield was shown to be unaffected by the salt.

The specific radioactivity of penicillin synthesized in the presence of the compounds tested was very similar to that in control experiments with L-[carboxy-¹⁴C]valine alone (column 5) and no marked decrease was observed. The low specific radioactivity of penicillin in one flask of Expt. no. 5 is not regarded as significant, particularly since penicillin from the duplicate flask had a slightly higher radioactivity than that in the control flasks. In Expt. no. 7 one of the two flasks also gave a low yield of penicillin and this result has been omitted from the comparison of the specific radioactivity of penicillin with that of the control (column 5). In both experiments, however, the results omitted would have given higher values for the mean specific radioactivity of penicillin than those given in column 5, and thus would not invalidate the general conclusion that addition of the test compounds did not decrease significantly the specific radioactivity of the synthesized penicillin.

Biological properties of the peptides. The possible antibiotic activity of the cyclic peptides (III) and (IV) was investigated by the cup-plate bioassay with *Bacillus subtilis* (Humphrey & Lightbown, 1952). No zones of inhibition were obtained with concentrations of 1 mg./ml. The phenylacetyl peptide (IV) has also been tested as a possible inducer of penicillinase, but was found to be inactive (Pollock, 1957).

DISCUSSION

Synthesis and properties of the cyclic peptides

Several cyclic peptides of a type similar to those synthesized in the present work have been reported previously in the literature, but cyclization has often been brought about by ring-closure of the thio ether rather than of the peptide bond. Thus Süs (1948) prepared the peptide (XIV; $R_1=H$;

$R_2 = C_6H_5 \cdot CH_2 \cdot CO$) by interaction of phenacetylpenicillamine with ethyl orthoformate, and Knunyants & Linkova (1955) converted *N*- β -chloropropionylpenicillamine into (XV, $R_1 = R_2 = CH_3$; $R_3 = CO_2H$) by hot methanolic baryta. However, attempts by Johnson (1956) to cyclize benzyloxycarbonyl- β -chloroalanylpenicillamine methyl ester to the benzyloxycarbonyl peptide (V) by a similar method gave the desired product only once and in poor yield (about 15%). This preparation could apparently not be repeated, and a comparison of the m.p. (190–210°) with that found in the present work (204°) suggests that the product may well have contained considerable amounts of an impurity.

Two other types of syntheses have also been reported: Sheehan & Cruickshank (1956*a, b*) obtained the methyl ester of (XIV; R_1R_2 = phthaloyl; or R_1 = H, R_2 = benzylsulphonyl) as by-products in the lactamization of the corresponding derivatives of 2-aminocarboxymethyl-4-methoxycarbonyl-5:5-dimethylthiazolidine, and the unsubstituted compound (XV; $R_1 = R_2 = R_3 = H$) has been prepared by ring enlargement of 1-thia-tetrahydro- γ -pyrone oxime (Panizzi, 1948).

In the present work, the cyclic cysteinylvaline peptides were obtained by cyclization of suitably protected monoazides of $\beta\beta$ -dimethyl-lanthionine, as outlined in Fig. 1. Synthesis of the acetyl peptide (III) was carried out first because the starting materials were most easily available. It was also hoped that the final product (III) might be deacetylated to the amino peptide (VI) without rupture of the peptide bond. The latter could undoubtedly be converted into the phenylacetyl derivative (V) without undue difficulty, but selective removal of the acetyl group from (III) proved impracticable. The phenylacetyl peptide (IV) which resembles penicillin most closely was prepared next for tests as a penicillin precursor. When this compound was found to be inactive it was decided to synthesize also the free amino peptide (VI), which involved preparation of the required intermediates protected by the benzyl-oxy-carbonyl group, which is easily removed by mild acid hydrolysis (Ben-Ishai & Berger, 1952).

The final cyclization of the azide, which took place in unexpectedly high yields, was based on the method previously used by Sheehan & Richardson (1954) for the preparation of cyclic 'triglycine', although it is now known that this product was in fact the dimeric cyclic hexaglycine (Bamford & Weymouth, 1955; Sheehan, Goodman & Richardson, 1955). In the present case, however, molecular-weight determinations of the peptides, as well as the absence of products other than monoacetyl-dimethyl-lanthionine (VII) when the acetylpeptide (III) was hydrolysed by acid under mild conditions,

indicate that dimerization to a 14-membered peptide did not occur to any significant extent. Molecular models show that the ring system in these peptides, unlike that in penicillin, is not strained because buckling of the ring is possible. On the other hand, the facile cleavage of the peptide bond by mild acid hydrolysis of (III) to (VII), and of (IV) to (VIII) (shown by paper chromatography only), suggests that this bond is unusually labile, particularly since cystinylvaline is completely hydrolysed by 6*N*-hydrochloric acid at 105° only after 72 hr. (H. R. V. Arnstein and D. Morris, unpublished experiments). It seems probable, therefore, that the seven-membered ring in the cyclic peptides is not entirely free of strain.

Penicillin biosynthesis

Possible precursor function of cyclic cystinylvaline peptides and derivatives of dimethyl-lanthione. The results in Table 2 show clearly that none of the added peptides or thio ethers related to cystine and valine were utilized for penicillin biosynthesis in preference to L-valine, since this would have resulted in some decrease in the specific radioactivity of the penicillin. Although it is difficult to estimate quantitatively the change in radioactivity to be expected in such isotopic competition experiments, an approximation can be made on the basis of the following calculation. Since addition of labelled L-valine at a concentration of 50 mg./l. gives rise to penicillin having about 30–50 % of the molar radioactivity of the added valine (Arnstein & Clubb, 1957), the total metabolic 'pool' of valine and penicillin intermediates is approximately 2–3 times that of the added valine, i.e. 1–1.5 m-moles/l. In the present experiments, the unlabelled compounds were added at a level of 250 mg./l. (0.7–1.1 m-moles/l., according to the actual compound tested) and the specific radioactivity of penicillin synthesized in the presence of the unlabelled precursor would therefore be expected to be not greater than $100 \times 1.5 / (0.7 + 1.5) = 67\%$ that in the control experiment without unlabelled precursor. In this calculation, however, no allowance has been made for the presence of two asymmetric centres in the cyclic peptides and dimethyl-lanthione derivatives; since only racemic products were used in this work the concentration of the correct stereoisomer will be only 25 % of the above figure, i.e. 0.2–0.3 m-mole/l. It would seem possible, therefore, that the specific radioactivity of penicillin produced could be as high as $100 \times 1.5 / (1.5 \times 0.2)$, i.e. 90 % of the control. It should be emphasized, however, that this represents the least favourable case; the metabolic 'pool' of an intermediate between valine and penicillin must be less than that of valine, since the last-named participates in the

biosynthesis of products other than penicillin, such as proteins.

On the most favourable assumption, on the other hand, the metabolic 'pool' of an intermediate of penicillin biosynthesis may be considered to be of the same order of magnitude as the amount of penicillin produced, i.e. about 7.3 μ g. (12 i.u.)/ml. (16 μ moles/l.) in the present experiments. The addition of 200 μ moles/l. of unlabelled intermediate to such a system would result in a marked decrease in the specific radioactivity of the penicillin, the expected radioactivity being $100 \times 16 / (16 + 200)$, i.e. 7.5 % of that of the control.

In practice neither of these extreme situations would be expected to apply and a reasonable assumption would be that utilization of the unlabelled compounds tested in the present work should result in a 20–25 % decrease in the specific radioactivity of the penicillin. Effects of this magnitude have been observed in similar isotope-competition experiments with ³⁵S-labelled sulphate in which L-cysteine was shown to be a penicillin precursor (Stevens, Vohra, Inamine & Roholt, 1953), although both labelled and unlabelled compounds were added at higher concentrations (3 m-moles/l.) than in the present experiments.

Mechanism of penicillin biosynthesis. The observation that $\beta\beta$ -dimethyl-lanthionine (II) does not decrease the conversion of labelled valine into penicillin confirms earlier isotopic-competition experiments by Stevens, Vohra, Moore & De Long (1954) in which ³⁵S-labelled sulphate or cystine were used as the radioactive components. Moreover, the monophenylacetyl derivative of dimethyl-lanthionine (VII), which contains the potential phenylacetyl side chain of penicillin, is apparently also not a precursor. This observation, together with earlier work which excluded the participation of phenylacetylcystine (Arnstein *et al.* 1954), indicates that neither of the following alternative pathways is involved in penicillin formation:

The first step in penicillin biosynthesis therefore probably involves synthesis of the peptide bond with formation of either cystinyl- or cysteinyl-valine. Since both the cyclic peptide (VI) and its phenylacetyl derivative (IV) are apparently excluded as intermediates, subsequent steps may consist of synthesis of the β -lactam ring followed by closure of the thiazolidine thio ether bond, for example by a mechanism similar to that suggested by Arnstein & Crawhall (1957). Although there is at present no direct evidence for such a pathway,

none of the present or earlier results is inconsistent with such a mechanism, which may thus serve as a working hypothesis for future experiments.

SUMMARY

1. The synthesis of 6-amino-2:2-dimethyl-5-oxo-hexahydro-1:4-thiazepine-3-carboxylic acid (cyclic DL-cysteinyl-DL-valine) and its acetyl and phenylacetyl derivatives is described.

2. The monoacetyl and monophenylacetyl derivatives of $\beta\beta$ -dimethyl-lanthionine, *S*-(2-acet-amido-2-carboxyethyl)-DL-penicillamine and *S*-(2-phenylacetamido-2-carboxyethyl)-DL-penicillamine respectively have also been prepared.

3. The compounds were tested as penicillin precursors by an isotope-competition method in which washed mycelium of *Penicillium chrysogenum* WIS 51-20 was incubated with L-[carboxy- ^{14}C]valine and the effect of addition of the unlabelled compounds on the specific radioactivity of the penicillin produced was measured.

4. None of the compounds was found to be an intermediate in the conversion of valine into penicillin. Phenylacetyl-L-cysteinyl-D-valine and $\beta\beta$ -dimethyl-lanthionine were also inactive as penicillin precursors.

5. Possible mechanisms of penicillin biosynthesis are discussed.

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The Metabolism of C_2 Compounds in Micro-organisms

1. THE INCORPORATION OF $[2-^{14}\text{C}]\text{ACETATE}$ BY *PSEUDOMONAS FLUORESCENS*, AND BY A *CORYNEBACTERIUM*, GROWN ON AMMONIUM ACETATE

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During the growth of micro-organisms, intermediates of the tricarboxylic acid cycle are utilized to provide the carbon skeletons of synthesized amino acids. When such growth occurs on complex substrates, carbon dioxide-fixing reactions, such as

form oxaloacetate or malate from pyruvate, are thought to replenish the supply of tricarboxylic acid cycle intermediates (Wiame & Bourgeois, 1955). Such reactions are not, however, sufficient to explain the processes by which micro-organisms