Blackburn, S. & Lee, G. R. (1955). *Analyst*, **80**, 875.
Blackburn, S. & Schöberl, A. (1956). *Hoppe-Seyl. Z.* **305**, 105.
Blackburn, S. & Tetley, P. (1956). *Biochim. biophys. Acta*, **20**, 423.
Greenstein, J. P. (1954). *Advanc. Protein Chem.* **54**, 121.
Holley, F. W., Peel, E. W., Luz, E. L. & Folkers, K. (1952). *J. Amer. chem. Soc.* **74**, 4539.
Popják, G. (1950). *Biochem. J.* **46**, 560.
Sheehan, J. C. & Hess, J. P. (1955). *J. Amer. chem. Soc.* **77**, 1067.
Stevens, C. M. & De Long, C. W. (1958). *J. biol. Chem.* **230**, 991.
Vaughan, J. R. (1951). *J. Amer. chem. Soc.* **73**, 3547.
Vaughan, J. R. & Eichler, J. A. (1953). *J. Amer. chem. Soc.* **75**, 5556.
Wood, J. R. & Du Vigneaud, V. (1939). *J. biol. Chem.* **130**, 109.

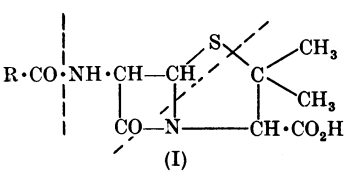
Biochem. J. (1960) **76**, 323

The Utilization of L-Cystinyl-L-Valine for Penicillin Biosynthesis

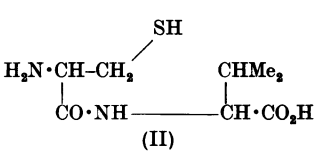
BY H. R. V. ARNSTEIN AND D. MORRIS
National Institute for Medical Research, Mill Hill, London, N.W. 7

(*Received* 21 *December* 1959)

There is now good evidence that the thiazolidine–β-lactam ring structure of penicillin (I) is derived from L-cystine and L-valine (for recent reviews see Demain, 1959; Arnstein, 1957) and oxidative ring-closure of the dipeptide cysteinylvaline (II) or of a closely related derivative has been suggested as a possible mechanism for the biosynthesis (Arnstein & Crawhall, 1957; Birch & Smith, 1958). In order to investigate the metabolism of this dipeptide in relation to penicillin biosynthesis, the disulphide form of (II) was synthesized from ^{14}C-labelled valine (Arnstein & Morris, 1960), both L-cystinyl-L-[*carboxy*-^{14}C]valine and L-cystinyl-D-[*carboxy*-^{14}C]valine being prepared.



(I)



(II)

R=$C_6H_5 \cdot CH_2 \cdot$ in benzylpenicillin. The broken lines indicate the metabolic origin of the thiazolidine–β-lactam ring structure from cysteine and valine.

In this paper it will be shown that *Penicillium chrysogenum* utilizes L-cystinyl-L-valine for penicillin formation without obligatory cleavage of the peptide bond. In contrast, the peptide containing D-valine was not taken up by the mycelial cells and appeared to be metabolically inert. Most of this work has been described by Arnstein & Morris (1959).

EXPERIMENTAL

Organism and maintenance of cultures. The strain of *Penicillium chrysogenum* used was WIS 51–20 F3, obtained from Dr W. J. Halliday, University of Queensland. Subcultures and spore inocula were prepared as described by Arnstein & Grant (1954).

Preparation of washed mycelium. Fermentations were carried out in the manner described by Halliday & Arnstein (1956) with the exception that the sterile potassium phenylacetate (0·1%/24 hr.) was added in two discrete portions after 24 and 40 hr. fermentations respectively. After 64–70 hr. the mycelium was harvested, washed three times by resuspension in 0·01 M-potassium phosphate buffer, pH 7, once by resuspension in 0·1 M-potassium phosphate buffer, pH 7, and filtered to give a mycelium of 13–15% dry wt.; the mycelium was then either used at once or stored at +1° under O_2 overnight for use on the following day. The potassium phosphate buffers referred to in this paper were prepared by diluting a M solution containing 136 g. of KH_2PO_4 and 40 g. of KOH/l.

Incubation of washed mycelium with precursors. Portions of mycelium (5 g. wet wt.) were added to duplicate 500 ml. conical flasks containing 50 ml. of ice-cold 0·01 M-potassium phosphate buffer, pH 7, 0·005% of potassium phenylacetate and 1% (w/v) of glucose (hereafter referred to as the medium) in the presence of a known quantity of ^{14}C-labelled precursor. After mixing at 0°, 1 ml. was removed from each flask, filtered and the filtrate was frozen and kept for uptake estimations (see below). The flasks were warmed rapidly to 25° and incubated on a rotary shaker (approx. 170 rev./min.) at this temperature for the required period. The flasks were then removed, chilled in ice and the medium was filtered by suction; 1 ml. of filtrate was removed from each flask for uptake estimation and frozen as before. The mycelial pads were immediately stored in solid CO_2 and the filtrate from each incubation was made up to 100 ml. in calibrated flasks with 0·1 M-potassium phosphate buffer, pH 7. After thorough mixing, portions (1 ml.) were

removed for bioassay and the solutions were made up to 100 ml. with 0·02 M-phosphate buffer, pH 7 (1 ml.), containing carrier sodium benzylpenicillin (50 mg.; potency 1670 i.u./mg.); after thorough equilibration of the contents the flasks were stored at 0° before isolation of the penicillin. In subsequent experiments conditions were altered slightly in that the incubation medium was warmed to 25° before adding the mycelium at the beginning of the experiment, and the filtrate at the end of the incubation was diluted to 50 ml., 75 mg. of carrier penicillin being added; the latter changes increased the efficiency of penicillin isolation.

Uptake estimations. The 1 ml. samples removed from each flask at the beginning and end of the incubation period were placed in a boiling-water bath for 2 min.; after cooling, a portion from each sample was placed on a 2 cm.^{2} polythene disk (containing lens tissue). The disks were dried *in vacuo* and weighed and counted. The counts were corrected to 'infinite thinness', with a self-absorption curve prepared with samples of known radioactivity plated under identical conditions. The percentage uptake was then calculated from the initial and final activities in the medium.

Penicillin bioassay. Penicillin was estimated by the cup–plate method with *Bacillus subtilis* (ICI strain N.C.T.C. 8241) as test organism (Humphrey & Lightbown, 1952).

Isolation of penicillin. Penicillin was extracted from the filtrate at 0° by acidification to pH 2 with 25% (v/v) phosphoric acid and extraction with ether. The volume of the solution was decreased by successive partitioning between ether at pH 2·0 and phosphate buffer at pH 7·0 (Smith & Hockenhull, 1952). The penicillin was isolated as the *N*-ethylpiperidine salt, which was purified to constant radioactivity by recrystallization from chloroform–acetone (1:4, v/v).

Radioactivity measurements. Unless otherwise stated, samples were mounted on 1 cm.^{2} polythene disks and counted at infinite thickness, as described by Arnstein & Clubb (1958).

Isolation of mycelial protein. The mycelium was defatted by boiling with ethanol and ether. The product was extracted with 5% (w/v) trichloroacetic acid (25 ml./g.) for 30 min. at room temperature, 15 min. at 90° and finally for 15 min. at room temperature. The residue was washed with ethanol and ether and dried *in vacuo* over P_2O_5. Samples were mounted on 1 cm.^{2} disks and counted as described above.

Labelled compounds. L-Cystinyl-L-[*carboxy*-^{14}C]valine and L-cystinyl-D-[*carboxy*-^{14}C]valine were synthesized as described by Arnstein & Morris (1959, 1960). The former peptide was isolated as the acetate salt. Uniformly ^{14}C-labelled L-valine, hereafter described as L-[U-^{14}C]valine, was obtained from The Radiochemical Centre, Amersham, Bucks.

Assessment of results. In order to facilitate assessment of the results, all specific activities have been corrected for differences of uptake and initial specific activities of precursors to give figures corresponding to 1 μC being available in the cells.

RESULTS

Incubation of the dipeptides with washed mycelium (Expt. 1)

Duplicate flasks containing L-cystinyl-L-[*carboxy*-^{14}C]valine acetate (0·49 μC, 5·9 mg.; equivalent to 0·0171 m-mole of valine), L-cystinyl-D-[*carboxy*-^{14}C]valine (0·49 μC, 4·7 mg.; equivalent to 0·0171 m-mole of valine) and L-[U-^{14}C]valine (0·215 μC, 2·5 mg.; 0·0171 m-mole) respectively were incubated with washed mycelium, in the medium described above, for 1, 2 and 4 hr. respectively. Uptake of the labelled precursors and incorporation of radioactivity into both penicillin and mycelial protein were determined. Uptake, expressed as the percentage of total activity available, is shown in Fig. 1. Good agreement (usually better than 2%) between duplicates was obtained and mean values have been plotted. At 1 hr. 69% of the added L-peptide had been taken up, but only 36% of the valine. After 2 hr. the uptake of L-peptide was virtually complete but that of valine rose to a similar figure only after a further 2 hr. No measurable uptake of D-peptide occurred and there was no radioactivity in either penicillin or mycelial protein in the experiments with this peptide.

Penicillin yields are shown in Table 1. Considering the incubations containing D-peptide as a control, penicillin production from L-peptide appeared to be very slightly inhibited in the early stages but was stimulated to some extent after 2 hr.; the reverse effect, only to a smaller extent, was observed in penicillin production from valine.

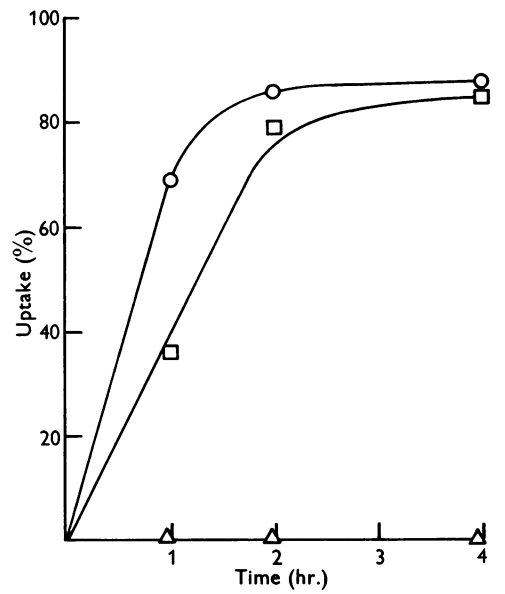


Fig. 1. Uptake of L-cystinyl-L-[*carboxy*-^{14}C]valine (○), L-cystinyl-D-[*carboxy*-^{14}C]valine (△) and L-[U-^{14}C]valine (□) into washed mycelium of *P. chrysogenum* WIS 51–20 F3 at 24° (see text, Expt. 1). Each point represents the mean percentage uptake of duplicate experiments.

The mean specific activities of penicillin *N*-ethylpiperidine salt and mycelial protein derived from incubations in the presence of L-peptide or L-valine for 1, 2 and 4 hr. respectively are shown in Fig. 2. At 1 hr. the peptide was more efficiently incorporated into penicillin than was valine; at 2 hr. the efficiencies were almost equal and at 4 hr. valine was incorporated more efficiently. At all times the specific activity of mycelial protein derived from peptide was considerably less than that from valine. The ratio of penicillin to protein activity was much higher at all times with peptide as precursor than it was with valine, as shown in Table 2.

Table 1. *Penicillin production by washed mycelium in the presence of* ^{14}C-*labelled valine and* ^{14}C-*labelled cystinylvaline*

In Expt. 1 (see text), washed mycelium of *P. chrysogenum* WIS 51–20 F3 was suspended in medium containing ^{14}C-labelled L-peptide acetate, D-peptide or L-[U-^{14}C]-valine. In Expt. 2, the experimental conditions were the same except that the D-peptide was not used and GSH (66 mg., 10 mol.prop.) was present. In Expt. 3, L-[U-^{14}C]-valine was incubated with and without GSH (66 mg., 10 mol.prop.) under the same conditions. Results are the means of duplicate flasks.

Expt.	Precursor	Time (hr.) 1	2	4
		Penicillin production (i.u./flask)		
1	L-Peptide	201	537	1260
	D-Peptide	226	491	976
	L-Valine	242	466	847
2	L-Peptide	192	338	—
	L-Valine	200	444	—
3	L-Valine (+GSH)	977	—	—
	L-Valine (control)	910	—	—

Table 2. *Comparison of the ratio of specific activities of penicillin and protein derived from* ^{14}C-*labelled* L-*cystinylvaline and* L-[U-^{14}C]*valine respectively*

For details of experiments see text.

Expt.	Incubation time (hr.)	Ratio of specific radioactivities: penicillin/protein L-Peptide as precursor	L-Valine as precursor
1	1	248	94
	2	96	55
	4	54	31
2	1	268*	104*
	2	136*	41·5*
3	1	—	146*
	1	—	109

* With glutathione in the medium.

Reduction of L-*peptide with glutathione*

It seemed possible that the peptide might be utilized more efficiently in the sulphydryl form, and reduction of the disulphide with excess of glutathione (GSH) was investigated as this appeared to be a convenient method. L-Cystinyl-L-[*carboxy*-^{14}C]valine acetate (5·56 mg., 88·8 μc/g.) was dissolved in 0·1 M-phosphate buffer, pH 7 (5 ml.), and diluted to 25 ml. with water. Glutathione (66 mg.; 10 mol.prop.) was dissolved in water (25 ml.) and the two solutions were mixed for 4 hr.; to each portion was added *N*-ethylmaleimide (0·4 ml., 10 mg./ml.) and the samples were freeze-dried. Chromatography on Whatman paper no. 1 in butanol–acetic acid–water (63:10:27, by vol.), followed by scanning for radioactivity on an automatic paper-chromatogram scanner, revealed that very rapid reduction of the peptide to the thiol form had occurred; no radioactivity was found at the R_F of cystinylvaline, a small amount was found at the origin and over 80% occurred at the R_F of authentic cysteinylvaline *N*-ethylmaleimide.

Incubation of precursors in the presence of glutathione (Expt. 2)

Duplicate flasks containing L-cystinyl-L-[*carboxy*-^{14}C]valine acetate (0·49 μc, 5·9 mg.; equivalent to 0·0171 m-mole of valine) or L-[U-^{14}C]valine (1·063 μc, 2·5 mg.; 0·0171 m-mole) were incubated, as described above, with washed mycelium for 1 and 2 hr. periods in the presence of GSH (66 mg., 10 mol.prop.). Bioassay figures are shown in Table 1.

The uptake of peptide was unaffected by the presence of GSH, the percentage uptakes at 1 and 2 hr. being 68·5 and 80% respectively, compared with 68·8 and 85·8% in Expt. 1 for corresponding

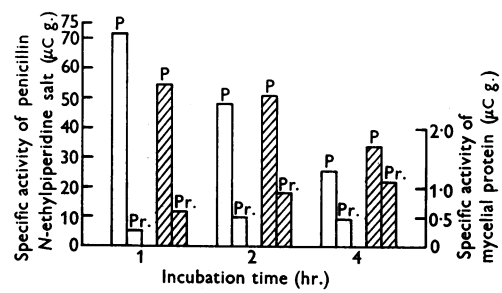


Fig. 2. Specific activities of penicillin *N*-ethylpiperidine salt and mycelial protein isolated after incubation of washed mycelium of *P. chrysogenum* WIS 51–20 F3 with L-cystinyl-L-[*carboxy*-^{14}C]valine acetate and L-[U-^{14}C]-valine respectively as described in the text (Expt. 1). Each value in the figure represents the mean of duplicate experiments. P, Penicillin; Pr., mycelial protein; unshaded and shaded areas refer to results from peptide and valine precursors respectively.

times; uptake of valine, however, was markedly decreased from 35·5 to 19·2% and from 78·8 to 51·6% respectively at the same times.

The specific activities of penicillin *N*-ethylpiperidine salt and mycelial protein are shown in the left-hand portion of Fig. 3. Compared with incubation without GSH (Expt. 1, Fig. 2), incorporation of peptide into penicillin was only slightly increased at 1 hr. but the increase was greater at 2 hr. Incorporation of valine, however, was practically doubled at 1 hr. but was relatively unaffected at 2 hr. Incorporation into protein with peptide as precursor was only slightly affected by GSH but a more significant increase occurred with valine as precursor. The ratios of specific activity of penicillin and mycelial protein are shown in Table 2. Once again the ratios with peptide as precursor were higher than with valine.

Incubation of L-[U-^{14}C]*valine with and without glutathione (Expt. 3)*

Duplicate flasks containing L-[U-^{14}C]valine (2·5 mg., 425 μc/g.) were incubated with washed mycelium for 1 hr. in the presence of GSH (66 mg., 10 mol.prop.); similar flasks without GSH were used as controls.

Bioassay figures are shown in Table 1. The overall penicillin yields were lower from this batch of washed mycelium but there was no significant effect of GSH on penicillin production.

Uptake of valine was decreased by GSH from 47·4 to 33·1%. The specific activities of penicillin and mycelial protein isolated are shown in the right-hand portion of Fig. 3. The increase in the specific activity of penicillin in the presence of GSH, observed in Expt. 2 (left-hand side of Fig. 3), was confirmed, although it was quantitatively not as great. Incorporation into mycelial protein was unaffected by GSH.

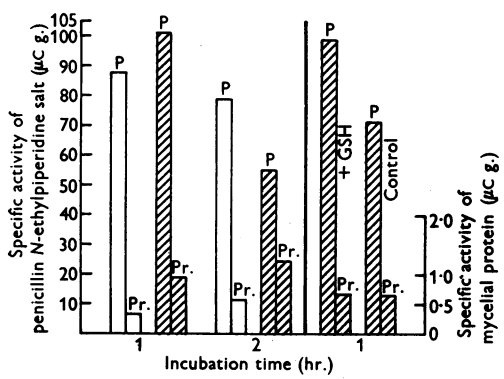


Fig. 3. Specific activities of penicillin *N*-ethylpiperidine salt and mycelial protein isolated after incubation of washed mycelium of *P. chrysogenum* WIS 51–20 F3 with L-cystinyl-L-[*carboxy*-^{14}C]valine acetate and L-[U-^{14}C]-valine in the presence of glutathione (Expt. 2); 10 equivalents of GSH with respect to peptide were used. The effect of glutathione on the incorporation of L-[U-^{14}C]valine (Expt. 3) is shown on the right-hand side. Conditions were as described in Fig. 2. P, Penicillin; Pr., mycelial protein; unshaded and shaded areas refer to results from peptide and valine precursors respectively.

DISCUSSION

In the present work it was found that L-cystinyl-D-valine is not metabolized by suspensions of washed mycelium of *P. chrysogenum* to any significant extent. Since this result may be due to lack of uptake of this compound by the mycelial cells, no conclusion is possible about the possible function of this peptide in penicillin biosynthesis.

On the other hand, L-cystinyl-L-valine was taken up by the mycelium even faster than L-valine and it appeared to be readily metabolized. Thus on the basis of the reasonable assumption that cystinyl-valine sequences in the mycelial protein would occur only rarely, the incorporation of radioactivity from the labelled peptide into protein indicates considerable hydrolysis into cystine and valine, although the finding that the specific radioactivity of the protein was considerably lower than in comparable experiments with labelled valine (Fig. 2) suggests that hydrolysis was not complete.

Compared with valine, the peptide is a better precursor of penicillin than of protein, as shown most clearly by the comparison of the ratios of the specific radioactivities of penicillin and protein (Table 2). It would appear therefore that despite extensive hydrolysis some of the peptide was converted into penicillin by a pathway not involving breakdown into its constituent amino acids, provided that free valine and valine released by hydrolysis of the peptide are equally available for penicillin and protein synthesis. The possible importance of such a pathway may be roughly assessed on the basis of the following considerations. Since the maximum rate of penicillin formation (Table 1) is approx. 300 units (0·5 μmole/flask/hr.) and the uptake of peptide in the first hour (Fig. 1) may be calculated to be about 12 μmoles, the labelled peptide is likely to be present in considerable excess compared with unlabelled peptide synthesized endogenously. Any extensive conversion of the peptide into penicillin would therefore be expected to give rise to a product having the same molar radioactivity as the labelled peptide. A comparison of the highest specific radioactivity of penicillin derived from peptide (72 μc/g. of *N*-ethylpiperidine salt, 32 μc/m-mole; see Fig. 2) with that of peptide available in the cell (83 μc/m-mole with respect to valine) shows that there was in fact dilution of the specific

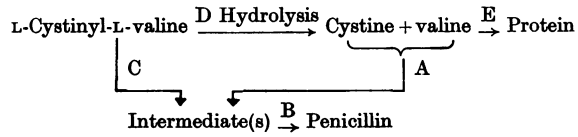
radioactivity. The direct conversion of L-cystinyl-L-valine into penicillin is thus relatively inefficient. It was thought that conversion of the peptide into the sulphydryl form might be rate-limiting, but reduction to L-cysteinyl-L-valine with excess of GSH did not result in any marked increase in the specific radioactivity of the penicillin, although GSH stimulated the utilization of valine to some extent.

compared with their utilization for protein synthesis.

2. L-Cystinyl-D-[*carboxy*-^{14}C]valine was not metabolized to any significant extent.

3. L-Cystinyl-L-[*carboxy*-^{14}C]valine was used preferentially for penicillin biosynthesis, indicating that this peptide can be utilized without hydrolysis into cystine and valine.

4. The extent of this direct utilization of



Scheme 1

These observations are summarized in Scheme 1. In this scheme, reactions D and E would lead to labelling of protein from cystinylvaline, whereas reaction C would account for the preferential incorporation of dipeptide into penicillin relative to protein, compared with that observed with valine as precursor. Penicillin biosynthesis would, however, take place mainly by pathways (A and B) involving intermediates other than L-cystinyl-L-valine or L-cysteinyl-L-valine. In this connexion it may be significant that the intracellular sulphur-containing amino acid and peptide fraction of the mycelium contains substantial amounts of δ-(α-aminoadipyl)cystinylvaline whereas cystinylvaline could not be detected (Arnstein, Morris & Toms, 1959). Full discussion of the possible significance of this tripeptide in penicillin biosynthesis (cf. Arnstein *et al.* 1959) will be deferred, but it may be noted that stepwise biosynthesis of this peptide starting from the *N*-terminal amino acid, as with GSH, would not require the formation of cystinyl-valine as an intermediate.

SUMMARY

1. The incorporation of radioactivity from L-cystinyl-L-[*carboxy*-^{14}C]valine, L-cystinyl-D-[*carboxy*-^{14}C]valine and uniformly labelled L-[^{14}C]-valine into penicillin by washed mycelium of *Penicillium chrysogenum* WIS 51–20 F3 has been

L-cystinyl-L-valine for penicillin formation appears to be quantitatively limited and the existence of a pathway of penicillin biosynthesis from cystine and valine, which does not involve cystinyl- or cysteinyl-valine as obligatory intermediates, is suggested.

We wish to thank Mr E. J. Toms for skilled technical assistance.

REFERENCES

Arnstein, H. R. V. (1957). *Rep. Progr. Chem.* **54**, 339.
Arnstein, H. R. V. & Clubb, M. E. (1958). *Biochem. J.* **68**, 528.
Arnstein, H. R. V. & Crawhall, J. C. (1957). *Biochem. J.* **67**, 180.
Arnstein, H. R. V. & Grant, P. T. (1954). *Biochem. J.* **57**, 353.
Arnstein, H. R. V. & Morris, D. (1959). *Biochem. J.* **71**, 8P.
Arnstein, H. R. V. & Morris, D. (1960). *Biochem. J.* **76**, 318.
Arnstein, H. R. V., Morris, D. & Toms, E. J. (1959). *Biochim. biophys. Acta*, **35**, 561.
Birch, A. J. & Smith, H. (1958). *Ciba Foundation Symp., Amino acids and peptides with antimetabolic activity*, p. 247. Ed. by Wolstenholme, G. E. W. & O'Connor, C. M. London: J. and A. Churchill Ltd.
Demain, A. L. (1959). *Adv. appl. Microbiol.* **1**, 23.
Halliday, W. J. & Arnstein, H. R. V. (1956). *Biochem. J.* **64**, 380.
Humphrey, J. H. & Lightbown, J. W. (1952). *J. gen. Microbiol.* **7**, 129.
Smith, E. L. & Hockenhull, D. J. D. (1952). *J. appl. Chem.* **2**, 287.