Table 1. Summary of the chromatographic and electrophoretic properties of the α - and γ -peptides

—, Signifies not determined.				
Chromatography*				
Solvent	R_F (disulphide form)		R_F ($-\text{SO}_3\text{H}$ form)	
	α -Peptide	γ -Peptide	α -Peptide	γ -Peptide
A	0.16	0.12	—	—
B	—	—	0.43	0.48
C	0.21	0.42	0.17	0.16
Electrophoresis† of $-\text{SO}_3\text{H}$ forms				
Migration (cm.)				
Conditions		Time	α -Peptide	γ -Peptide
2N-Acetic acid, pH 2.1; 25 v/cm./26 ma		4 hr.	3.0	3.0
0.35M-Pyridine-acetic acid, pH 3.5; 19 v/cm./14 ma		1 hr.	9.0	13.0
0.35M-Pyridine-acetic acid, pH 3.5; 66 v/cm./24 ma		20 min.	2.3	5.6
* Whatman no. 1 paper.		† Whatman 3MM paper.		

was concentrated *in vacuo* and freeze-dried to give crude α -L-glutamyl-L-cystinyl-L-valine (XIV, 600 mg.). Chromatography in solvent A revealed the presence of two main spots, R_F 0.16 (major) and R_F 0.11 (minor), the latter being identical with that of γ -peptide marker; very small traces of other spots were also found. Chromatography in solvent C gave a similar picture: major spot, R_F 0.21; minor spot, R_F 0.42 (γ -peptide marker gave R_F 0.41). The crude peptide (530 mg.) was crystallized from aqueous ethanol to give chromatographically pure material (200 mg., R_F 0.16 in solvent A); one further crystallization, followed by drying at 110° for 2 hr., gave analytically pure α -L-glutamyl-L-cystinyl-L-valine dihydrate m.p. > 300°, $[\alpha]_D^{25}$ - 12.2° (c, 0.98 in 5N-HCl) (Found: C, 42.9; H, 6.6; N, 11.4. $\text{C}_{27}\text{H}_{44}\text{O}_{13}\text{N}_6\text{S}_2 \cdot 2\text{H}_2\text{O}$ requires C, 42.6; H, 6.6; N, 11.4%).

A summary of the chromatographic and electrophoretic properties of the α - and γ -peptides in both their disulphide and oxidized ($-\text{SO}_3\text{H}$) forms is given in Table 1.

SUMMARY

1. The synthesis of the α - and γ -forms of L-glutamyl-L-cystinyl-L-valine is reported.

2. The limitations of the methods used are described.

I should like to thank Dr H. R. V. Arnstein for helpful advice and encouragement, and to express my appreciation to Mr E. J. Toms for skilled technical assistance.

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Sulphur-Containing Amino Acids and Peptides in the Mycelium of *Penicillium chrysogenum*

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Washed mycelium of *Penicillium chrysogenum* is able to utilize L-cystinyl-L-valine for penicillin

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biosynthesis by a pathway not involving breakdown to the free amino acids (Arnstein & Morris, 1960b). It is possible, however, that some further modification of the dipeptide may be necessary before the latter is incorporated into penicillin, and

therefore the mycelium of *P. chrysogenum* has been examined for the presence of cystinylvaline, or of modified peptides containing both cystine and valine. This paper describes the isolation of such a peptide, containing α -amino adipic acid. This work has been reported briefly elsewhere (Arnstein, Morris & Toms, 1959).

EXPERIMENTAL

Methods and materials

Preparation of washed mycelium. Fermentation of *P. chrysogenum* WIS 51-20 F3 was carried out as described by Halliday & Arnstein (1956), but in the absence of phenylacetic acid. After 63 hr. the fermentation was stopped and the mycelium was harvested by filtration. The mycelium was washed three times by suspension in 0.01 M-buffer and filtered to give a mycelium of 13% wet wt.; the mycelium was used at once.

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).

Paper chromatography. Preliminary work indicated the necessity of using washed papers. Whatman 3MM papers were washed successively (by descending solvent flow in a chromatography tank) with M-ammonium acetate, M-acetic acid, water and ion-exchange water. The papers were dried in an oven and checked for removal of peptides and amino acid by chromatography (solvent B) of a 0.3 M-NH₃ soln. eluate of a sample strip of paper. The solvent systems used had the following compositions (by vol.): solvent A, butanol-acetic acid-water, 63:10:27; solvent B, phenol-water, 5:2 (with NH₃ in the tank); solvent C, pyridine-water, 4:1. Where necessary, chromatograms were developed with ninhydrin (0.1% in butanol containing 2% of water).

Paper electrophoresis. Washed Whatman 3MM paper was used unless otherwise stated. Pyridine-acetic acid buffers were prepared as follows: (a) pH 3.5 buffer, a mixture of acetic acid (100 ml.) and pyridine (10 ml.) diluted to 1 l. with water; (b) pH 5 buffer, acetic acid (105 ml.) and pyridine (140 ml.) diluted to 1 l. with water. The quantities mentioned give 1.75 M-buffers, which were diluted appropriately before use. The stated concentration refers to that of the anionic component.

Radioactivity measurements. Radioactivity on paper chromatograms was located and measured on a two-dimensional scanner. Radioactivity of materials in solution was determined, after evaporation of samples on to 2 cm.² polythene disks containing lens tissue, with a thin end-window Geiger-Müller tube. Measurements of radioactivity were made mainly for detection of labelled materials and were not intended to be a basis for strictly quantitative estimation.

Radioactive compounds. Uniformly labelled L-[¹⁴C]valine was obtained from The Radiochemical Centre, Amersham, Bucks.

Unlabelled compounds. γ -L-Glutamylcysteylvaline and cysteylvaline were obtained by performic acid oxidation of the disulphide form of the peptides (Morris, 1960; Arnstein & Morris, 1960a); γ -glutamylcysteylglycine (GSO₂H) was similarly obtained from glutathione (GSH). $\beta\beta$ -Dimethylcysteic acid (penicillaminic acid) was pre-

pared either by degradation of benzylpenicillin to penicilloic acid, followed by performic acid oxidation, or by oxidation of penicillamine.

Incubation of washed mycelium with L-[¹⁴C]valine. In order to facilitate the subsequent isolation of metabolic products derived from valine, washed mycelium was incubated with L-[¹⁴C]valine for 1 hr. as described below. Portions (10 g. wet wt.) of mycelium (giving 10 units of penicillin/ml./hr.) were added to each of six Erlenmeyer flasks (500 ml.) containing medium (100 ml.) of the following composition: 0.01 M-phosphate buffer, pH 7, glucose (1%, w/v), potassium phenylacetate (0.005%, w/v) and L-[¹⁴C]valine (5 mg., 8.3 μ C). The flasks were incubated on a rotary shaker (140 rev./min.) for 1 hr. at 24°. After incubation the contents of all six flasks were combined and filtered. The mycelium was washed with 0.01 M-phosphate buffer, pH 7.

Extraction of amino acids and peptides from mycelium

The mycelium obtained above was transferred immediately to boiling aqueous ethanol (70%, v/v; 200 ml.) and boiled for 10 min. The suspension was filtered and the residue on the filter was boiled once more with ethanol (70%; 100 ml.). The combined filtrates were cooled at 0° and centrifuged and the sediment was treated again with ethanol. All the ethanol extracts were combined and concentrated *in vacuo* until all the ethanol had been removed. The aqueous solution was centrifuged and the supernatant solution was applied to a column of Dowex-50 resin (12% cross-linked, 200-400 mesh, Microchemical Specialties Co., Berkeley, Calif., U.S.A.) of dimensions 75 cm. $\times$ 1.8 cm. diam. The column was eluted with water until the eluate was neutral and the amino acids and peptides were displaced from the column with 0.3 M-NH₃ soln. (700 ml.). The alkaline eluate was concentrated *in vacuo* below 40° to 250 ml. and then freeze-dried to give the crude desalted amino acid-peptide mixture.

Removal of non-sulphur-containing amino acids and peptides

The amino acid-peptide mixture obtained after the desalting step was treated with performic acid (10 ml. of 98% formic acid + 3 ml. of 100 vol. H₂O₂) at room temp. for 1 hr. to oxidize cystine and cystine-containing peptides to the corresponding sulphonc acids. After addition of water (100 ml.) the reaction mixture was freeze-dried. The residue was dissolved in water (20 ml.) and loaded on to a Dowex-50 column (75 cm. $\times$ 1.8 cm. diam.) as before. The acidic sulphur-containing materials were eluted with water until the eluate was neutral. (Measurement of radioactivity in a sample of further eluate showed that the elution was complete.) The eluate was freeze-dried and its radioactivity was determined. This fraction was usually contaminated with a trace of valine, which was, however, completely removed by subsequent chromatography on paper with solvent B (see below), as shown in a control experiment with a mixture of labelled valine and γ -glutamylcysteylvaline.

Fractionation of S-containing amino acids and peptides

Samples of the mixture were streaked in bands (usually 30 cm.) on washed Whatman 3MM paper and chromato-

graphed in solvent *B*. Cysteic acid, cysteiclyvaline, γ -glutamylcysteiclyvaline, GSO_3H and penicillaminic acid were used as markers. After running, the paper was dried and radioactive bands were located by scanning. Non-radioactive bands were detected by ninhydrin staining of thin strips cut from either end of the streaked area. Both active and inactive bands were cut out and eluted with 0.3M- NH_3 soln.; the rest of the chromatogram was developed with ninhydrin to locate the markers. The eluted bands were then subjected separately to paper electrophoresis at 700 v in 2N-acetic acid for 6 hr. The various bands were detected as before and the composition of the components was established either by comparison with authentic materials or by hydrolysis. Further purification, when necessary, was achieved by chromatography in solvent *C*, two-dimensional chromatography in solvents *A-C*, or by electrophoresis at 66 v/cm. in 0.35M-pyridine-acetic acid, pH 3.5 or pH 5.

Quantitative estimation of peptide hydrolysates

A sample of peptide, the constituent amino acids of which had been determined previously by hydrolysis, was hydrolysed in 6N-HCl at 105° for 18 hr. The acid solution was evaporated to dryness twice with water; the residue was subjected to two-dimensional analysis on Whatman no. 3 paper by electrophoresis at 3.5 kv in 0.175M-pyridine-acetic acid, pH 5, and chromatography in solvent *A*. After the first dimension, quantitative markers of the constituent amino acids, in an appropriate range of concentrations, were applied to the paper. After the second dimension, the concentrations of the peptide-hydrolysate amino acids were determined by comparison with the standard markers, by the method of Mandelstam & Rogers (1959).

RESULTS

Isolation of S-containing amino acids and peptides

The crude, desalted, mycelium extract obtained from the first column had a radioactivity of 11.5 μC and weighed 575 mg. After oxidation the sulphur-containing materials weighed 189 mg. and the activity was 0.4 μC .

Fractionation of S-containing material

Chromatography. Paper chromatography in solvent *B* resolved the mixture into two ninhydrin-positive bands. Band P (R_f 0.07) was non-radioactive and had the same R_f as both the cysteic acid and GSO_3H markers. Band Q (R_f 0.20) was radioactive (activity was estimated to be 0.14 μC), with the same R_f as both γ -glutamylcysteiclyvaline and penicillaminic acid.

Electrophoresis. Band P was resolved into two components (P_1 , P_2) with migrations identical with those of GSO_3H and cysteic acid respectively. Band Q was also resolved into two components (Q_1 , Q_2) with migrations identical with those of γ -glutamylcysteiclyvaline and penicillaminic acid respectively. Both components were radioactive.

Identification of components

P_1 was inferred to be GSO_3H both from its chromatographic and electrophoretic behaviour and by the release of glutamic acid, cysteic acid and glycine on hydrolysis. P_2 had properties identical with those of cysteic acid.

Component Q_2 was identified as penicillaminic acid ($\beta\beta$ -dimethylcysteic acid) by its chromatographic behaviour described above. The presence of radioactivity was shown to be due to the fact that it was derived from penicillin by the isolation and oxidation procedure and hence labelled after incubation with [^{14}C]valine (Halliday & Arnstein, 1956); treatment of ^{14}C -labelled penicillin in the same way as described for the isolation of substances containing amino acids and peptides produced a radioactive compound identical in properties with Q_2 .

A sample of Q_1 ran as one radioactive spot, with properties very similar to γ -glutamylcysteiclyvaline (I), on two-dimensional chromatography in the solvent system *C-A*, namely: solvent *C* (R_f 0.44; marker R_f 0.41), solvent *A* (R_f 0.07; marker R_f 0.065). Elution and hydrolysis of this material gave cysteic acid, glycine, glutamic acid, a spot with the same R_f as alanine and [^{14}C]valine (two-dimensional chromatography in solvent *A*). A further sample of Q_1 , after chromatography in solvent *C* followed by elution, hydrolysis and examination of the hydrolysate by two-dimensional chromatography in solvent system *C-A*, gave a hydrolysis pattern identical with that described above. This result suggested that Q_1 was possibly either a pentapeptide or a smaller peptide contaminated with either GSO_3H or adsorbed amino acids. The first possibility seemed unlikely in view of the virtually identical chromatographic properties of Q_1 and (I). On electrophoresis at pH 3.5 (to remove any adsorbed neutral amino acids before hydrolysis) Q_1 behaved as one spot, with a migration identical with that of (I). Hydrolysis of a portion of Q_1 , which had been further purified by electrophoresis at pH 5.0, gave mainly valine, cysteic acid and α -amino adipic acid, together with a small amount of glutamic acid and a trace of glycine, all identified by two-dimensional electrophoresis (pH 5)-chromatography in solvent *A*. Alanine had the same R_f as α -amino adipic acid in solvents *A* and *C* but did not migrate on electrophoresis at pH 5.0.

Quantitative examination of the hydrolysate (see Experimental section) showed that Q_1 is a tripeptide containing α -amino adipic acid, cysteic acid and valine (Table 1, Expt. 1). The amount of glycine obtained in the first experiment is very small relative to valine, whereas in control experiments with blank paper glutamic acid was found to be eluted even from washed paper; acid hydrolysis

Table 1. *Result of quantitative hydrolysis of tripeptide isolated from mycelium of Penicillium chrysogenum*

Amino acids were estimated on paper by the method of Mandelstam & Rogers (1959). Expt. 1 refers to peptide containing [^{14}C]valine. Expt. 2 (a): peptide contaminated with GSO_3H . Expt. 2 (b): result of further purification before hydrolysis.

Expt. no.	Amino acids	Yield (μmole)	Molar ratio	Estimated total yield of peptide ($\mu\text{g./g. of fresh mycelium}$)
1	Valine	0.062	1.0	5.4
	α -Aminoadipic acid	0.058	0.93	
	Cysteic acid	0.060	0.97	
	Glutamic acid	0.014	0.23	
	Glycine	0.007	0.11	
2a	Valine	0.053	1.0	3.0
	α -Aminoadipic acid	0.050	0.95	
	Cysteic acid	0.085	1.60	
	Glutamic acid	0.039	0.74	
	Glycine	0.035	0.66	
2b	Valine	0.018	1.0	—
	α -Aminoadipic acid	0.025	1.39	
	Cysteic acid	0.029	1.61	
	Glutamic acid	0.002	0.11	
	Glycine	0.004	0.22	

of such eluates indicated the presence of glutamic acid also in bound form, which appears to be adsorbed on to other compounds during chromatography. Repeated running of Q_1 on paper during the isolation procedure could well result in contamination by the amount of glutamic acid found in the first experiment. Glutamic acid was confirmed as an artifact when the structure of the peptide was determined (Arnstein & Morris, 1960c). In a second experiment with peptide isolated from mycelium which had not been incubated with radioactive valine, Q_1 appeared to be contaminated with adsorbed GSO_3H in the ratio of 1.0:0.7 (see Expt. 2a, Table 1), although the bulk of the GSO_3H had been well separated. Further purification of the peptide decreased this contamination to less than 10%, as shown by the relative proportions of glutamic acid and α -aminoadipic acid (Table 1, Expt. 2b). In this estimation the proportion of valine relative to cysteic acid and α -aminoadipic acid is low, probably because the accuracy of the method was reduced by the small amounts available for this analysis compared with the others.

The quantitative estimation of α -aminoadipic acid after acid hydrolysis of the peptide may be used to calculate the amount of peptide which had been extracted from the mycelium. This calculation indicates that the peptide is present in amounts of at least 3–5 $\mu\text{g./g.}$ wet wt. of mycelium (Table 1).

Some evidence was also obtained for the presence of a similar or identical peptide in extracts of a commercial strain of *P. chrysogenum*, grown in the presence of a side-chain precursor of penicillin, but the quantity was too small for full investigation.

DISCUSSION

Several investigations of sulphur compounds produced by *Penicillium chrysogenum* have been reported. Tardrew & Johnson (1958) compared the distribution of sulphate and organic sulphur in the mycelium and of penicillin and total sulphur excreted into the medium, and showed that the addition of a precursor of the penicillin side chain resulted in an increased excretion of penicillin but in a decrease in the organic-sulphur content of the mycelium. The latter finding suggests that penicillin formation depletes intracellular stores of sulphur compounds and our failure to isolate appreciable amounts of peptide from a high-penicillin-producing commercial strain may thus have been due to the presence of side-chain precursor.

Among the sulphur compounds isolated from mycelium, methionine has been obtained in relatively large amounts (approx. 1 mg./g. of mycelium) (Gordon, Numerof & Pan, 1954) and another major constituent is choline sulphate (De Flines, 1955), which appears to be present in similar amounts (2 mg./g. dry wt., Stevens & Vohra, 1955). Choline sulphate has been shown not to be an intermediate in penicillin biosynthesis (Stevens & Vohra, 1955). Both these compounds would not have been isolated by the method used in the present work, since choline sulphate is not retained by cation-exchange resins (De Flines, 1955), whereas methionine would have been adsorbed even after oxidation to the sulphone.

A minor constituent of the intracellular-sulphur fraction of *P. chrysogenum* is known to be penicillin

itself (Demain, 1957), which accounts for the presence of penicillaminic acid ($\beta\beta$ -dimethylcysteic acid) in the oxidized sulphur amino acid and peptide fraction. On the assumption that fermentations contain about 10% of fresh mycelium, it may be calculated from the results of Demain (1957) that mycelium contains about $0.3 \mu\text{mole}$ of penicillin/100 g. fresh wt., compared with a total organic-sulphur content of $200 \mu\text{moles}$ and $370 \mu\text{moles}$ /100 g. fresh wt. in the presence and absence of side-chain precursor respectively (Tardew & Johnson, 1958). The amount of α -aminoadipic acid present as the peptide described in this paper corresponds to approx. $0.9\text{--}1.6 \mu\text{moles}$ /100 g. fresh wt. of mycelium. This tripeptide should therefore be considered another minor constituent of the intramycelial-sulphur fraction. The foregoing amounts are, however, comparable with those of penicillin and hence not incompatible with a function of the peptide as an intermediate in penicillin biosynthesis. By inspection of the paper chromatograms it was clear that glutathione was present in much larger (possibly 10–50-fold) amounts than the peptide, although no quantitative estimation was made. No evidence could be obtained, however, for the presence in the mycelium of cystinylvaline or any other peptide containing both cystine and valine, and a function of the aminoadipic acid peptide in penicillin biosynthesis is therefore not unlikely.

SUMMARY

1. *Penicillium chrysogenum* WIS 51–20 F3 was incubated briefly with [^{14}C]valine and extracts of the mycelium were examined for the presence of

peptides containing cystine and valine, as well as for related sulphur compounds.

2. The isolation of a tripeptide which contains α -aminoadipic acid, cysteic acid [formed from cyst(e)ine during the isolation procedure, which involved oxidation with performic acid] and valine is described.

3. After oxidation with performic acid mycelial extracts also contained cysteic acid, penicillaminic acid (derived from penicillin) and oxidized glutathione (GSO_3H).

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The Structure of a Peptide, Containing α -Aminoadipic Acid, Cystine and Valine, Present in the Mycelium of *Penicillium chrysogenum*

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In an investigation of possible intracellular intermediates in penicillin biosynthesis, a peptide containing cystine, valine and α -aminoadipic acid was isolated from the mycelium of *Penicillium chrysogenum* (Arnstein, Artman, Morris & Toms, 1960). This paper deals with a study of the amino acid sequence of this peptide and with the occurrence of free α -aminoadipic acid in *P. chrysogenum*. The possible implications of these results for penicillin

biosynthesis will also be discussed. A preliminary note of these observations has been published (Arnstein, Morris & Toms, 1959).

EXPERIMENTAL

Methods

Paper electrophoresis. Electrophoretic investigations were carried out on Whatman 3MM paper with a high-