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 (Tardrew & 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-

voltage apparatus capable of supplying up to 10 kv at 100 ma. The paper was cooled by close contact with a water-cooled insulated support.

Radioactivity measurements. Rough estimates of radioactivity were made for qualitative or semi-quantitative purposes either by direct counting on paper with an automatic two-dimensional scanner, or by counting of chromatogram eluates, after evaporation on to 2 cm.² polythene disks, with a thin end-window Geiger-Müller tube.

Preparation of 2:4-dinitrophenyl derivatives. 2:4-Dinitrophenyl (DNP) derivatives were prepared by the method of Sanger & Thompson (1953). Dinitrophenol was removed at 60° and 0.1 mm. Hg pressure in a vacuum-sublimation apparatus.

Estimation of dinitrophenyl derivatives. The relative amounts of DNP partial-degradation products were estimated by measurement of their absorption at 360 mμ with a Hilger u.v.-absorption spectrophotometer.

Buffer solutions. Buffer concentrations are stated with respect to the anionic component. Phosphate buffers were made from sodium salts.

Materials

Cysteicvaline, γ-glutamylcysteicvaline and α-glutamylcysteicvaline were obtained by performic acid oxidation of the corresponding cystine peptides (Arnstein & Morris, 1960a; Morris, 1960). α-Aminoadipic acid and DNP-α-aminoadipic acid were kindly given by Dr E. P. Abraham, University of Oxford.

Determination of the N-terminal amino acid

A sample of the radioactive peptide (Arnstein *et al.* 1960) (370 counts/min., equivalent to approx. 9 μg. of valine residue) was converted into the DNP derivative and hydrolysed with 6N-HCl (1 ml.) at 110° for 18 hr. The acid solution was extracted three times with ether (3 ml. portions); all the yellow colour passed into the ether layer. The ethereal solution was evaporated to dryness *in vacuo* and the residue (in 50 μl. of ethanol) was applied to buffered paper (pH 6) and examined by two-dimensional chromatography in *tert.*-amyl alcohol-phosphate buffer (pH 6) (Blackburn & Lowther, 1951; Levy, 1954). The paper was run for 5 days in the first dimension against markers of DNP derivatives of aspartic acid, glutamic acid, glycine, valine and cysteic acid; the second dimension was run for 5 hr. against markers of DNP-glutamic acid and DNP-aspartic acid respectively.

A mixture of DNP-aspartic acid, DNP-glutamic acid and DNP-α-aminoadipic acid was subjected to electrophoresis at 86 v/cm. in 0.08M-pyridine-acetic acid buffer, pH 5, for 1 hr., in a preliminary experiment. The unknown DNP-amino acid was eluted from the two-dimensional chromatogram, dried *in vacuo*, and the residue was subjected to high-voltage electrophoresis under the conditions just described; the three DNP-dicarboxylic amino acids were used as markers.

The unknown DNP-amino acid was eluted once again and an approximately equal amount of authentic DNP-α-aminoadipic acid was added. The mixture was then subjected to high-voltage electrophoresis at 80 v/cm. in 0.175M-pyridine-acetic acid buffer, pH 5, for 1 hr. against DNP-α-aminoadipic acid and DNP-glutamic acid, present both singly and as a mixture.

Partial-acid-hydrolysis experiments

γ-Glutamylcysteicvaline (1.5 mg.) was converted into the DNP-derivative in the usual way. Anal. R 12N-HCl (0.3 ml.) was added and the mixture was placed in a stoppered siliconed test tube at 37°. Samples (30 μl.) were removed after 29, 46 and 70 hr., and, after drying *in vacuo* over P₂O₅-KOH, were examined by paper electrophoresis at 20 v/cm. in 0.08M-pyridine-acetic acid, pH 5, for 1.5 hr. The degradation products were examined for structure by comparison with markers, both before and after development with ninhydrin, or by further degradation. The hydrolysis time for maximum concentration of partial-hydrolysis products was determined by comparison of the absorption of these products in solution at 360 mμ.

A sample of the unknown tripeptide (approx. 80 μg., as estimated by radioactivity) was converted into the DNP derivative as before. The product was subjected to electrophoresis at 80 v/cm. in 0.08M-buffer, pH 5, for 1 hr. to check homogeneity and was then eluted from the paper with water (0.65 ml.). A portion (0.1 ml.) was evaporated on to a 2 cm.² disk and counted at infinite thinness. The DNP-peptide solution was evaporated to dryness and then treated with 12N-HCl (0.5 ml.) at 37° for 48 hr. The hydrolysate was evaporated to dryness twice with water to remove excess of HCl and the residue was subjected to electrophoresis at 46 v/cm. in 0.08M-buffer (pH 5), for 2 hr. Valine, cysteicvaline, cysteic acid, DNP-α-aminoadipic acid and DNP-γ-glutamylcysteicvaline were used as markers. The degradation products were examined for structure as before. In addition, the relative concentrations of DNP products were estimated after elution from the paper by their absorption at 360 mμ, and radioactive spots were detected by scanning and then counted after elution and evaporation on to 2 cm.² disks.

Isolation of α-aminoadipic acid from extracts of the mycelium of *Penicillium chrysogenum*. After elution of cysteic acid peptides and other acidic substances from the second column of Dowex-50 cation-exchange resin used for the isolation of sulphur-containing peptides and amino acids (Arnstein *et al.* 1960), the eluent was changed from water to 1.5N-HCl. Fractions (20 ml.) were collected and analysed by evaporating portions (0.3 ml.) to dryness *in vacuo* and examining the residue by paper chromatography (phenol-water, 5:2, v/v, with NH₃ in the tank) and paper electrophoresis (0.08M-pyridine-acetic acid, pH 5, 2 hr. at 25 v/cm.). Fractions nos. 53-63, which contained α-aminoadipic acid together with traces of glutamic acid and a neutral amino acid (probably glycine), were combined and freeze-dried. This material was further fractionated on a column of De-Acidite FF resin (25 g.; The Permutit Co. Ltd., London) in the acetate form. 0.5M-Acetic acid was used as eluent and 5 ml. fractions were collected. Only two fractions (nos. 6 and 7) contained pure α-aminoadipic acid, but chromatography of the adjacent fractions (5 and 8) again on the same column yielded more pure material. α-Aminoadipic acid was identified by comparison by paper electrophoresis and chromatography as described above. In addition, butanol-acetic acid-water (63:10:27, by vol.) was used as a second solvent for paper chromatography. The fractions containing pure α-aminoadipic acid were combined and freeze-dried. The solid was dissolved in 5N-HCl (1 ml.); the rotation of this solution was determined and the amount of α-aminoadipic acid was estimated

by quantitative paper chromatography (Mandelstam & Rogers, 1959), butanol-acetic acid-water (63:10:27, by vol.) being used as the chromatographic solvent ($[\alpha]_D +14.3 \pm 5.7^\circ$; c , 0.175). The observed rotation corresponds to a molecular rotation of $+23.1 \pm 9.2^\circ$; the literature value for L- α -aminoadipic acid (c , 0.5-2%) is $+40.3^\circ$ (Greenstein, 1954).

Examination of the mycelial protein and cell-wall fraction for the presence of α -aminoadipic acid. Mycelium of *Penicillium chrysogenum* WIS 51-20 F3 was defatted with boiling ethanol, ethanol-ether (3:1, v/v) and ether and extracted with hot 5% trichloroacetic acid. The extracted protein-rich residue (60 mg.) was hydrolysed with 6N-HCl (0.6 ml.) for 18 hr. at 105° . After addition of a little water the hydrolysate was filtered and evaporated to dryness *in vacuo* over KOH. The residue was dissolved in water (0.4 ml.) and portions (10 μ l., equivalent to 1.5 mg. of extracted protein + cell-wall fraction) were examined by electrophoresis on Whatman no. 3MM paper (0.08M-pyridine-acetic acid, pH 5, 53v/cm. for 75 min.), amino acids being detected by spraying with 0.1% ninhydrin in butanol containing 2% of water. Good separation of α -aminoadipic acid, glutamic acid and aspartic acid markers was obtained, but the hydrolysate contained only glutamic acid and aspartic acid. Since less than 1.5 μ g. of α -aminoadipic acid is visible, the presence of 0.1% of α -aminoadipic acid in the extracted mycelium would have been detected by this procedure.

RESULTS

N-terminal amino acids

The unknown DNP-amino acid ran as one spot in both dimensions on two-dimensional chromatographic analysis. In the first dimension it was not clearly separated from DNP-glutamic acid or DNP-aspartic acid. In the second dimension, however, the material moved slightly slower than DNP-glutamic acid, which itself was slower than DNP-aspartic acid. This result suggested that the unknown was probably a higher homologue of these acids, for example, α -aminoadipic acid. In so far as the peptide had already been shown to consist of one residue of each of cysteic acid, valine and α -aminoadipic acid (Arnstein *et al.* 1960), the last-named was almost certainly *N*-terminal. Preliminary electrophoresis of DNP-glutamic acid, DNP-aspartic acid and DNP- α -aminoadipic acid showed that these compounds were completely separated at pH 5. Under the same conditions the unknown material migrated 17.1 cm. (identical with that of DNP- α -aminoadipic acid marker) and was clearly not DNP-aspartic acid (migration 22.5 cm.). The migration of the two DNP-glutamic acid markers, however, was not identical (16.5 and 18.2 cm.), presumably caused by uneven wetting of the paper. In the final experiment the mixture of the unknown and DNP- α -aminoadipic acid ran as one spot, whereas the mixture of DNP- α -aminoadipic acid and DNP-glutamic acid separated into two distinct

components. These results demonstrated conclusively that the *N*-terminal amino acid of the tripeptide was α -aminoadipic acid.

Partial-hydrolysis experiments

Partial hydrolysis of DNP- γ -glutamylcysteylvaline gave three yellow components, corresponding, in order of increasing electrophoretic mobility, to DNP-glutamic acid, unchanged DNP-peptide and an unknown DNP product. The latter was shown, by elution, hydrolysis and subsequent paper chromatography in butanol-acetic acid-water (63:10:27), to be DNP- γ -glutamylcysteyl acid. After development with ninhydrin three blue spots were seen in addition to the three yellow components described above. The ninhydrin-positive spots were shown, by comparison with markers, to be valine, cysteic acid and cysteic acid respectively. The order of increasing migration of the six degradation products was valine, cysteic acid, DNP- γ -glutamylcysteylvaline and DNP- γ -glutamylcysteyl acid.

The $\epsilon_{\max}$ values of DNP- γ -glutamylcysteyl acid at 29, 46 and 70 hr. were 0.06, 0.070 and 0.058 respectively, indicating that a hydrolysis time of about 46 hr. was optimum for maximum production of this degradation product.

Electrophoresis of the partial hydrolysate of the oxidized natural peptide gave a very similar result to that described above. The electrophoresis pattern is shown in Fig. 1. The six degradation

Fig. 1. Electrophoresis of DNP-peptide for 2 hr. at 3.5 kv in 0.08M-pyridine-acetic acid buffer, pH 5, after treatment with 12N-HCl for 48 hr. at 37° . Result is shown in column B. Markers: column A (in order of increasing mobility), valine, cysteic acid, DNP- α -aminoadipic acid and cysteic acid; column C, DNP- γ -glutamylcysteylvaline. The paper was scanned for radioactivity and then developed with ninhydrin. For identification of the products numbered in parentheses, see text. Y, Yellow; B, blue; R, radioactive.

products were: (1) valine, (2) cysteiclylvaline, (3) DNP- α -aminoadipic acid, (4) cysteic acid, (5) unchanged DNP-peptide, (6) DNP- α -aminoadipyl-cysteic acid, as shown below. Valine and cysteiclylvaline were identified by their mobility compared with authentic specimens and by the presence of radioactivity (detected by scanning). DNP- α -aminoadipic acid had the same mobility as the authentic marker, and the unchanged DNP-peptide was radioactive and had a mobility identical with that of DNP- γ -glutamylcysteiclylvaline. The structure of compound (6) was inferred from its mobility, which was very similar to that of DNP- γ -glutamylcysteic acid, and from the absence of radioactivity, showing that valine had been lost from the starting material. Products (5) and (6), after they had been eluted, evaporated to dryness and taken up in water (0.5 ml.), had $\epsilon_{\max}$ values at 360 $m\mu$ of 0.495 and 0.620 respectively; the radioactivity contained in these products was 103.2 and 6.3 counts/min. respectively. Since (5) is present in smaller amount than (6) and yet has more than 16 times the radioactivity, the very small activity in (6) can be neglected, thus confirming the absence of valine. These results indicate that the amino acid sequence of the tripeptide is α -aminoadipyl-cysteiclylvaline.

Electrophoresis of free tripeptide

The free peptide had an electrophoretic mobility (3.0 cm.), identical with the mobilities of α - and γ -glutamylcysteiclylvaline at pH 2.1. At pH 3.5, however, the α -glutamyl tripeptide had a migration of 4.2 cm., whereas both the γ -glutamyl and aminoadipyl tripeptides had an increased and identical migration of 10.0 cm. This behaviour shows that, analogously to the γ -glutamyl peptide, the α -carboxyl group of the α -aminoadipic acid is free and therefore the latter must be linked to the rest of the molecule by its δ -carboxyl group. Thus the

nately, insufficient material was available for determination of the optical configuration of the constituent amino acids. The possible biogenetic relationship of (I) to cephalosporin N (II, Newton & Abraham, 1954) is, however, sufficiently indicated by the similarity in the amino acid sequence and by the linkage of the α -aminoadipic acid through its δ -carboxyl group in both compounds. If the D-configuration of the valine moiety of the penicillin and cephalosporin molecule is introduced during cyclization, as has been suggested previously (Arnstein & Crawhall, 1957; Birch & Smith, 1958), the valine residue in (I) should be in the L-configuration. Free α -aminoadipic acid, which has been found in the extracts of mycelium together with the peptide, appears to be present predominantly as the L-enantiomorph, although the very

small amount available considerably decreased the accuracy of measurement of the optical rotation. Since the α -aminoadipic acid side chain of cephalosporin N has the D-configuration (Newton & Abraham, 1954), α -aminoadipic acid in (I) could be either the D- or the L-form depending on whether the optical configuration is changed before or after cyclization.

A possible pathway for penicillin biosynthesis is summarized in the following scheme (Cys, cystine or cysteine; Val, valine; Aad, aminoadipic acid):

structure of the tripeptide isolated from mycelial extracts after oxidation with performic acid must be δ -(α -aminoadipyl)cysteiclylvaline.

DISCUSSION

The evidence presented in this paper indicates that the structure of the α -aminoadipic acid peptide which occurs in the mycelium of *P. chrysogenum* is either (I) or its disulphide. Unfortun-

This scheme would account for the presence of (I) in amounts comparable with the intracellular concentration of penicillin (Arnstein *et al.* 1960) and for the direct utilization of labelled cyst(e)inylvaline for penicillin biosynthesis (Arnstein & Morris, 1960b), presumably by incorporation of the labelled dipeptide into (I) by a transpeptidation reaction similar to that involved in the incorporation of cysteinylglycine into glutathione (Fodor, Miller, Neidle & Waelsch, 1953). By analogy with

glutathione, new synthesis of (I) would, however, take place with the intermediary formation of α -aminoadipylcystine, which would account for the observation that the conversion of labelled cystinylvaline into penicillin was quantitatively limited (Arnstein *et al.* 1960).

Cyclization of (I) would give rise to cephalosporin N, which has, however, not yet been detected in fermentations of *P. chrysogenum* (see Arnstein, 1957). Since cephalosporin N is postulated only as an intermediate, it would be expected to be present in very small amounts, possibly only intracellularly, and a careful investigation of penicillin-like compounds in the mould mycelium would therefore be of interest. The final step of penicillin biosynthesis would involve exchange of the α -aminoadipic acid side-chain of cephalosporin N for a carboxylic acid, e.g. phenylacetic acid with benzylpenicillin. Such a reaction seems not unlikely in view of the known side-chain transfer between benzyl- and phenoxymethyl-penicillin (Peterson & Wideburg, 1958). It is also suggested that the mould *Cephalosporium*, which synthesizes cephalosporin N, but is unable to produce benzylpenicillin even in the presence of phenylacetic acid (see Arnstein, 1957), may lack the appropriate transferase. On the other hand, 6-aminopenicillanic acid, which is biosynthesized by *Penicillium chrysogenum* in the absence of phenylacetic acid or other side-chain precursors (Batchelor, Doyle, Nayler & Rolinson, 1959), may arise by loss of the side chain of cephalosporin N and would therefore be a shunt product rather than an intermediate in penicillin biosynthesis.

Although there are still many gaps in the evidence for this scheme of penicillin biosynthesis, experimental verification of most of its implications would appear to be feasible without undue difficulty.

SUMMARY

1. The *N*-terminal group of a peptide which occurs in the mycelium of *Penicillium chrysogenum* has been shown to be α -aminoadipic acid and the

amino acid sequence has been determined by partial hydrolysis with acid.

2. The structure of the peptide is δ -(α -aminoadipyl)cyst(e)inylvaline.

3. α -Aminoadipic acid has also been isolated from mycelium and appears to be present mainly as the *L*-enantiomorph.

4. The possible function of α -aminoadipic acid, of the peptide and of cephalosporin N in penicillin biosynthesis is discussed.

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