Biosynthesis of Peptides containing α -Aminoadipic Acid and Cysteine in Extracts of a *Cephalosporium* sp.

By P. BRONWEN LODER AND E. P. ABRAHAM

Sir William Dunn School of Pathology, University of Oxford, Oxford OX1 3RE, U.K.

(Received 15 March 1971)

1. Three intracellular peptides found in small amount in a *Cephalosporium* sp. were rapidly labelled when DL-[14 C]valine was added to a shaken suspension of the organism. More 14 C was incorporated into peptide P3, δ -(L- α -aminoadipyl)-L-cysteinyl-D-valine, than into peptide P2 (containing α -aminoadipic acid, cysteine, valine and glycine) or peptide P1 (containing β -hydroxyvaline in place of the valine in peptide P2). 2. Peptides P3 and P2, but not peptide P1 were formed in a broken-cell system from the *Cephalosporium* sp. in the presence of δ -(L- α -aminoadipyl)-L-cysteine and DL-[14 C]valine. No synthesis was observed in the presence of δ -(D- α -aminoadipyl)-L-cysteine or of DL- α -amino[14 C]adipic acid and L-cysteinyl-L-valine or L-cysteinyl-D-valine. 3. The biosynthesis of these peptides was catalysed by the particulate fraction of the broken-cell system, whereas that of glutathione was catalysed by the supernatant fraction. 4. These results are discussed in relation to penicillin N and cephalosporin C biosynthesis.

A peptide complex (Aad-Cys-Val* complex) extracted from the mycelium of a *Cephalosporium* sp. behaved like δ -(α -aminoadipyl)cysteinylvaline on oxidation with performic acid and subsequent electrophoresis on paper at pH 1.8. This complex was labelled from [14 C]valine and α -amino[14 C]adipic acid when these amino acids were added to a shaken suspension of washed mycelium (Warren, Newton & Abraham, 1967a,b). Further investigation showed that the complex could be resolved into three peptides, P1, P2 and P3. Peptide P3 was δ -(L- α -aminoadipyl)-L-cysteinyl-D-valine, peptide P2 contained α -aminoadipic acid, cysteine, valine and glycine and peptide P1 contained α -aminoadipic acid, cysteine, β -hydroxyvaline and glycine (Loder & Abraham, 1971).

Experiments on the possible role of δ -(α -aminoadipyl)cysteine as an intermediate in the biosynthesis of penicillin N and cephalosporin C showed that the dipeptide did not enter the mycelium intact but was hydrolysed to its constituent amino acids, probably near the cell surface (Loder, Abraham & Newton, 1969). The present paper describes the results of a study of the biosynthesis of the peptides of the Aad-Cys-Val complex by intact mycelium and by an extract of the *Cephalosporium* sp.

EXPERIMENTAL

Materials. Pyruvate kinase and phosphoenolpyruvate (tricyclohexylammonium salt) were obtained from Boehringer Corp. (London) Ltd., London W.5, U.K.

*Abbreviation: Aad, α -aminoadipic acid.

ATP was obtained from the Sigma (London) Chemical Co. Ltd., London S.W.6, U.K. DL-[1- 14 C]Valine (32.2 mCi/mmol), [1- 14 C]glycine (41.4 mCi/mmol), and DL- α -amino-[1- 14 C]adipic acid (13.8 mCi/mmol) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. The disulphide of δ -(L- α -aminoadipyl)-L-cysteinyl-L-valine was kindly supplied by Professor Rudinger. *N*-Benzyloxycarbonyl-*S*-benzyl-L-cysteine was from BDH Chemicals Ltd., Poole, Dorset, U.K. δ -(L- α -Aminoadipyl)-L-cysteine, the corresponding D- α -aminoadipyl isomer and γ -L-glutamyl-L-cysteine were prepared as described by Loder *et al.* (1969).

General methods. Paper chromatography and electrophoresis, radioautography and the counting of radioactive compounds on planchets and on paper were carried out as described by Smith, Warren, Newton & Abraham (1967). Determinations of protein were made by the method of Lowry, Rosebrough, Farr & Randall (1951). Thiol groups were determined by the method of Ellman (1959) and detected on paper by spraying with starch-iodine (Sneath & Collins, 1961). Oxidation of thiol compounds to the corresponding sulphonic acids with performic acid was carried out as described by Smith *et al.* (1967).

Synthesis of cysteinylvaline. *S*-Benzyl-*N*-benzyloxycarbonyl-L-cysteine (1.72 g) was coupled with L-valine ethyl ester (0.75 g) by a mixed-anhydride method as described for its coupling with L-alanine methyl ester by MacLaren, Savige & Swan (1958). The product was obtained as an oil (2.1 g). This ethyl ester was dissolved in dioxan (15 ml), 3M-NaOH (6 ml) was added and the mixture shaken for 10 min. Water (12 ml) was added followed by sufficient dioxan to produce a single phase. After 1 h the solution was brought to pH 5 with 4M-HCl and evaporated to dryness *in vacuo*. The residue was dissolved in ethyl acetate (15 ml) and shaken first with M-NaOH (5 ml) and then with water (10 ml). On acidification of the water extract to pH 2 with 11M-HCl an oil

precipitated which solidified after keeping for 16 h at 4°C. The supernatant was decanted and the crude *S*-benzyl-*N*-benzyloxycarbonyl-L-cysteinyl-L-valine washed with water and dried *in vacuo* (1.1 g).

The protective groups were removed from this product by treatment with Na in liquid NH₃ in an apparatus similar to that of Nesvadba & Roth (1967). After removal of the NH₃ the residue was dissolved in 0.25 M-H₂SO₄ (20 ml) and the solution extracted twice with ether. Ether was removed from the aqueous layer *in vacuo* and the mercury salt of the dipeptide was precipitated by the addition of the HgSO₄-H₂SO₄ reagent of du Vigneaud & Miller (1952). The mercury salt was washed twice with boiled water by centrifugation, suspended in boiled water (15 ml) and decomposed with H₂S. Excess of H₂S was removed from the supernatant *in vacuo*, the solution was adjusted to pH 3.0 with saturated Ba(OH)₂, and the precipitated BaSO₄ removed by centrifugation. The supernatant was freeze-dried to yield 480 mg of crude dipeptide.

A portion of the crude product (60 mg) was dissolved in ethanol containing 0.1% acetic acid (2 ml) and applied to a column (95 cm × 1.5 cm diam.) of Sephadex LH20. Elution was carried out with the same solvent and fractions (2 ml) collected every 5 min. Fractions 75–85, which contained the dipeptide uncontaminated with cysteine or cystine, were combined and the resulting solution concentrated to a small volume and freeze-dried after dilution with water. The resulting hygroscopic white solid (37 mg) consisted of L-cysteinyl-L-valine partly in the form of its sulphate.

This preparation of L-cysteinyl-L-valine behaved as a single substance when subjected to electrophoresis at pH 1.8 and located by either the ninhydrin or starch-iodine spray, migrating 1.4 times as far as cysteine towards the cathode. When chromatographed in butan-1-ol-acetic acid-water (4:1:4, by vol.) it was not resolved from cysteine. After oxidation with performic acid it migrated 0.1 times as far as glutathionesulphonic acid towards the anode.

L-Cysteinyl-D-valine was made by the same procedure and behaved in a similar manner. Both preparations yielded only valine and cysteine (or cystine) on hydrolysis with 6 M-HCl for 42 h at 105°C.

Suspensions of washed mycelium. *Cephalosporium* sp. C91 was harvested 72 h after inoculation and the mycelium was separated and washed as described by Smith *et al.* (1967). The mycelium was resuspended in boiled water (1 g of damp-dry mycelium/6 ml) and portions (5 ml or 25 ml) dispensed into shake-flasks (50 ml or 250 ml) which were shaken on a rotary shaker (200 rev./min and 2 in throw) at 27.5°C.

Incubation of [¹⁴C]valine and [¹⁴C]glycine with suspensions of washed mycelium. DL-[1-¹⁴C]Valine (50 μCi, 1.55 μmol) or [1-¹⁴C]glycine (50 μCi, 1.21 μmol) were added to suspensions of washed mycelium in water (25 ml) which had been shaken for 30 min at 27.5°C. After a further 15 min or 60 min shaking at 27.5°C, the extracellular fluid was removed by filtration and the mycelium washed well with water. The mycelial pellet was shaken for 5 min with 12.5 ml of ice-cold 70% (v/v) ethanol and for 1 min with 12.5 ml of ice-cold water. The ethanol and water extracts were combined, evaporated to small volume in a rotary evaporator, and freeze-dried. The residue was

dissolved in water (2 ml), the solution adjusted to pH 3 with M-HCl and insoluble material removed by centrifugation. The supernatant was applied to a column (10 cm × 1 cm diam.) of Dowex 50 (X4; H⁺ form; 200–400 mesh). The column was washed with water until the effluent was free of ¹⁴C, and then amino acids and peptides were eluted with m-pyridine. The eluate was freeze-dried and the residue oxidized with performic acid. The oxidized material was dissolved in water (1 ml), and applied to a column (5 cm × 1 cm diam.) of Dowex 50 (X4; H⁺ form; 200–400 mesh) which was then washed with water (15 ml) and fractions (2 ml) collected. Percolate fractions containing peptide-sulphonic acids were combined and freeze-dried. Samples corresponding to one-fifth or one-tenth of the total were analysed by two-dimensional paper electrophoresis and chromatography, followed by radioautography and coloration with ninhydrin.

Incubation of cysteinylvaline with suspensions of washed mycelium. Freshly prepared solutions of L-cysteinyl-L-valine and L-cysteinyl-D-valine were added to suspensions in water of washed mycelium in shake-flasks at 27.5°C to give a final concentration of peptide of 0.7 μmol/ml. Estimations of the sulphhydryl content of the extracellular fluid, and also of control solutions containing the dipeptides in boiled water, were made at 30 min intervals. After 3 h the extracellular fluid was removed by filtration and freeze-dried. The mycelial pellet was washed well with water, then shaken for 5 min with 2.5 ml of ice-cold 70% (v/v) ethanol and for 1 min with 2.5 ml of ice-cold water. These ethanol and water extracts were combined and freeze-dried. The extracellular material and the mycelial extract were analysed by paper electrophoresis and chromatography before and after performic acid oxidation.

Ultrasonic treatment of mycelium. Washed mycelium was resuspended in 0.1 M-tris-HCl buffer, pH 7.0 (containing 0.1 M-KCl, 0.02 M-MgSO₄ and 0.001 M-cysteine), and the suspension (1 g of damp-dry mycelium/5 ml water) subjected to ultrasonic treatment as described by Smith *et al.* (1967). Part of the suspension was centrifuged for 30 min at 20000g and the supernatant removed. The latter contained about 3.7 mg of protein/ml. The particulate portion was washed once by resuspension in buffer (20 ml) and centrifugation at 20000g and finally resuspended in buffer to give a final volume equal to that of the whole ultrasonically treated preparation.

Synthesis of glutathione and the 'Aad-Cys-Val complex' in broken-cell systems. [1-¹⁴C]Glycine (5 μCi, 0.12 μmol) was added to portions of the various extracts together with γ-L-glutamyl-L-cysteine and glycine, or with L-glutamic acid, L-cysteine and glycine, to give final concentrations of amino acids and peptide of 4 μmol/ml. DL-[1-¹⁴C]Valine (5 μCi, 0.15 μmol) was added to portions of the various extracts together with 8-(α-amino adipyl)-L-cysteine and L-valine, or with L-α-amino adipic acid, L-cysteine and L-valine (final concentrations 4 μmol/ml). DL-α-Amino[1-¹⁴C]adipic acid (5 μCi, 0.35 μmol) was added together with L-cysteinylvaline and L-α-amino adipic acid, or with L-cysteine, L-valine and L-α-amino adipic acid (final concentrations 4 μmol/ml). The specific radioactivity of the L-[¹⁴C]valine and L-α-amino[1-¹⁴C]adipic acid present in the incubation mixtures was thus 0.6 μCi/μmol. Incubations were carried out in stoppered tubes at 27.5°C for 1 h, in the presence of an energy-generating system, which was as follows: ATP, 5 mM;

phosphoenolpyruvate, 9mM; pyruvate kinase, 0.25mg/ml; KCl, 0.1M; MgSO₄, 0.02M (final concentrations). All acidic amino acids and peptides were neutralized with m-NaHCO₃ before addition to the extracts.

Preparation of products for paper chromatography and electrophoresis. At the end of the incubation period the mixture (0.5–1.0ml) was centrifuged at 20000g for 30 min and the supernatant freeze-dried. For further analysis the residue was desalted. In most cases residues were extracted with 70% (v/v) ethanol (2×200μl), and the ethanol extracts diluted with water and freeze-dried. Half of the freeze-dried product was oxidized with performic acid as described by Smith *et al.* (1967). Some residues were dissolved in water (1ml) and the solution applied to a column (5cm×1cm diam.) of Dowex 50 (X4; H⁺ form; 200–400 mesh). The column was washed with water (6ml) and peptides and amino acids were eluted with m-pyridine. The eluate was freeze-dried and half of the residue oxidized with performic acid. Comparisons indicated that the two desalting procedures yielded similar results.

RESULTS

Biosynthesis of intracellular peptides by intact mycelium

The amounts of ¹⁴C incorporated into the components of the Aad-Cys-Val complex and into glutathione when suspensions of washed mycelium were shaken in water after the addition of DL-[1-¹⁴C]valine (2μCi/ml, 0.062μmol/ml) or [1-¹⁴C]glycine (2μCi/ml, 0.048μmol/ml) are given in Table 1. The major part of the ¹⁴C incorporated into the Aad-Cys-Val complex from DL-[¹⁴C]valine was found in peptide P3 and after 15 min accounted for about 0.2% of the total radioactivity added. The labelling of this peptide had passed its maximum by 60 min and may have reached it before 15 min. Peptide P2 was labelled, as expected, from [¹⁴C]glycine as well as from DL-[¹⁴C]valine. On the assumption that only one optical isomer of the added DL-valine was used the results indicated that both amino acids were incorporated with similar efficiency. The amount of ¹⁴C incorporated into peptide P1 from DL-[¹⁴C]valine was about twice

as much as that incorporated into peptide P2, but [¹⁴C]glycine appeared to be incorporated into peptide P1 with somewhat lower efficiency.

Analysis of the extracellular fluid and the intracellular pool of the mycelium after L-cysteinyl-L-valine or L-cysteinyl-D-valine had been added to a mycelial suspension in water in shake-flasks showed that both dipeptides were oxidized extracellularly to the corresponding disulphides, the reaction being virtually complete within 3h, and that there was no detectable hydrolysis or uptake of the dipeptide by the mycelial cell.

Biosynthesis of peptides in broken-cell systems

The ability of various broken-cell systems to synthesise glutathione and the Aad-Cys-Val complex from [¹⁴C]glycine and [¹⁴C]valine respectively in the presence of an energy-generating system is shown in Table 2. There was no close correspondence between the conditions under which the two types of peptide were formed. Labelled glutathione was formed mainly in the supernatant fraction of an ultrasonic extract, whereas labelled Aad-Cys-Val complex, on the other hand, was synthesized in the whole ultrasonic extract and a suspension of the particulate fraction, but not in the supernatant (Expt. II).

With the whole extract the radioactivity in the Aad-Cys-Val complex was only about 10% of that in glutathione. [¹⁴C]Glutathione was formed from L-glutamic acid, L-cysteine and [¹⁴C]glycine and its synthesis was not greatly increased by the addition of γ-L-glutamyl-L-cysteine (Expt. I). Separate experiments showed that the amount of glutamic acid and cysteine in the sonic extract itself were sufficient for synthesis to occur at a maximum rate under the conditions used. In contrast, the synthesis of the Aad-Cys-Val complex from [¹⁴C]valine was only detected in systems to which δ-(L-α-amino-adipyl)-L-cysteine had been added. No labelled complex was found after the addition of the corresponding D-α-aminoadipyl peptide or of free

Table 1. *Incorporation of ¹⁴C from [¹⁴C]valine and [¹⁴C]glycine into intracellular peptides by washed mycelium*

Four flasks (250 ml) each containing 25 ml of mycelial suspension were shaken for 30 min. DL-[1-¹⁴C]Valine (50μCi, 32.2μCi/μmol) and [1-¹⁴C]glycine (50μCi, 41.4μCi/μmol) were each added separately to two flasks. The mycelium from two flasks was harvested after 15 min and from the remaining two flasks after 60 min. For details and methods of analysis see the Methods section.

Addition	Time (min)	¹⁴ C found (nCi) in sulphonic acids of peptides			
		P1	P2	P3	GSH
DL-[1- ¹⁴ C]Valine	15	2.8	1.2	114	
	60	2.0	1.0	41.2	
[1- ¹⁴ C]Glycine	15	1.0	1.5	—	1383
	60	0.8	2.5	—	1129

Table 2. *Synthesis of cysteine-containing peptides in broken-cell systems of the Cephalosporium sp.*

Experiments with different numbers were with different batches of mycelium. The final concentration of added amino acids and dipeptides was 4 mM and the total volume was 1.2 ml. The specific radioactivity of added [^{14}C]glycine was $1.25\mu\text{Ci}/\mu\text{mol}$. The specific radioactivity of added [^{14}C]-L-valine and [^{14}C]-L- α -aminoadipic acid was $0.6\mu\text{Ci}/\text{mol}$. Incubations were for 1 h at 27.5°C . In none of the experiments was radioactivity detected in substance P1. Abbreviations: Aad-Cys, δ -(α -aminoadipyl)-L-cysteine; Cys-Val, L-cysteinylvaline; Glu-Cys, γ -L-glutamyl-L-cysteine; Aad, α -aminoadipic acid. For details of the systems and analytical procedures see the text.

Expt.	Extract	Addition	Radioactivity (nCi) found in			
			GSH	Aad-Cys-Val complex	Peptide P2	Peptide P3
I	Complete system	[^{14}C]Val + L-Aad-Cys		3.4	1.1	1.1
		[^{14}C]Val + L-Aad + L-Cys		<0.1		
		[^{14}C]Gly + L-Glu-Cys	21.8			
		[^{14}C]Gly + L-Glu + L-Cys	16.6			
II	Complete system	[^{14}C]Val + L-Aad-Cys		1.4	0.7	0.7
		[^{14}C]Gly + L-Glu-Cys	10.0			
	Supernatant	[^{14}C]Val + L-Aad-Cys		<0.1		
		[^{14}C]Gly + L-Glu-Cys	12.0			
	Particulate fraction	[^{14}C]Val + L-Aad-Cys		2.2	0.7	0.8
		[^{14}C]Gly + L-Glu-Cys	3.8			
III	Complete system	[^{14}C]Aad + L-Cys-L-Val		<0.1		
		[^{14}C]Aad + L-Cys-D-Val		<0.1		
		[^{14}C]Aad + L-Cys + L-Val		<0.1		
IV	Complete system	[^{14}C]Val + L-Aad-Cys		4.1	1.5	1.5
		[^{14}C]Val + D-Aad-Cys		<0.1		
		[^{14}C]Val + L-Aad + L-Cys		<0.1		

L- α -aminoadipic acid and L-cysteine. Further, no labelled complex was detected after the addition of DL- α -amino[^{14}C]adipic acid and L-cysteinyl-D-valine or L-cysteinyl-L-valine.

The labelled Aad-Cys-Val complex, which in the sulphonic acid form appeared as a single spot after electrophoresis at pH 1.8, was analysed further by chromatography in a second dimension in butan-1-ol-acetic acid-water. In each case two spots with similar radioactivities were found which coincided with ninhydrin-positive spots from added sulphonic acids of peptides P2 and P3 (Loder & Abraham, 1971). No labelled peptide P1 was detected in these experiments, nor was any observed when [^{14}C]glycine ($1.25\mu\text{Ci}/\mu\text{mol}$; final concentration 4 mM) was added to a complete ultrasonic extract together with δ -(L- α -aminoadipyl)-L-cysteine and DL- β -hydroxyvaline (each 4 mM).

In experiments with whole ultrasonic extracts of mycelium from cultures which produced relatively high amounts of penicillin N and cephalosporin C, members of the Aad-Cys-Val complex were the only cysteine-containing peptides labelled from DL-[^{14}C]valine. With extracts from other batches of mycelium a new labelled substance (P4) appeared which in the sulphonic acid form migrated only about 0.3 times as far as the sulphonic acid of peptide P3 towards the anode at pH 1.8. Unlike

peptide P3 or peptide P2, this material was labelled in extracts to which only amino acids and no dipeptide were added. The radioactivity in peptide P4 reached its maximum with an extract from a batch of mycelium that had produced an abnormally low yield of penicillin N (8 units/ml instead of about 30 units/ml). With the extract from this batch of mycelium no Aad-Cys-Val formation was detected, but a substance (P5) was labelled from [^{14}C]valine which migrated slightly less far towards the anode than Aad-Cys-Val on electrophoresis at pH 1.8 and was not resolved from the latter. When chromatographed on paper in butan-1-ol-acetic acid-water after electrophoresis, peptide P5 was resolved into two labelled components with R_F values of 1.4 and 1.6. The structures of peptide P4 and of the components of peptide P5 have not been determined.

DISCUSSION

Although the broken-cell system from the *Cephalosporium* sp. which was used in these experiments may have contained some viable cells (Smith *et al.* 1967) the latter could not have been responsible for the biosynthesis of the peptides that was observed, since the corresponding peptides that they produce remain entirely intracellular (Warren *et al.* 1967b).

The finding that peptide P3 was δ -(L- α -amino-

adipyl)-L-cysteinyl-D-valine (Loder & Abraham, 1971) was consistent with the observation that δ -(α -aminoadipyl)-cysteinylvaline was formed in a broken-cell system from δ -(L- α -aminoadipyl)-L-cysteine and DL-[14 C]valine, but not from the corresponding D- α -aminoadipyl dipeptide; but whether L- or D-valine, or both, are used in the biosynthesis of the tripeptide remains to be determined.

If this tripeptide is a precursor of the penicillin N produced by the *Cephalosporium* sp. the amino acids of the corresponding tripeptide isolated earlier by Arnstein & Morris (1960) from *Penicillium chrysogenum* are likely to have the same optical configuration. The latter organism produces isopenicillin N, which has a δ -(L- α -aminoadipyl) side chain in place of the δ -(D- α -aminoadipyl) side chain of penicillin N and a D-penicillamine (β -thiolvaline) fragment that is common to penicillin N and other penicillins (Flynn, McCormick, Stamper, De Valeria & Godzeski, 1962; Cole & Batchelor, 1963). The D-configuration of the side chain of the extracellular penicillin N would then arise at a late stage in the biosynthetic pathway. A very small amount of an antibiotic with the chromatographic properties of penicillin N has been found in the intracellular pool of the mycelium of the *Cephalosporium* sp. (Smith *et al.* 1967; Warren *et al.* 1967a, b), but whether this has a D or L- α -aminoadipyl side chain is not known.

The presence of a D-valine residue in peptide P3 raises the question whether the D-configuration of the penicillamine fragment of the penicillin ring system originates from L-valine during synthesis of the tripeptide and not, or not exclusively, during the formation of the thiazolidine ring by addition of -S- to a dehydrovaline fragment as suggested earlier by Arnstein & Crawhall (1957). The finding that peptide P1 contains a residue of β -hydroxyvaline would be consistent with this mechanism, since the thiazolidine ring could be formed from a β -hydroxyvaline residue in a manner analogous to that by which cystathionine is produced from homoserine and cysteine (Kaplan & Flavin, 1966). However, Stevens & Halpern (1949) found no β -hydroxyvaline in the mycelium of *P. chrysogenum* and Arnstein & Clubb (1957) showed that free β -hydroxyvaline was not an effective precursor of the penicillamine fragment of benzylpenicillin. These findings indicate that if a β -hydroxyvaline residue is involved as an intermediate in penicillin biosynthesis it is formed from valine after the latter has been incorporated into a peptide intermediate. This would be consistent with the failure to observe labelling of peptide P1 from [14 C]glycine in a broken cell system to which δ -(L- α -aminoadipyl)-L-cysteine and DL- β -hydroxyvaline had been added.

Alternatively, the formation of a peptide containing α -aminoadipic acid, cysteine and β -

hydroxyvaline might represent a diversion from the main pathway to the β -lactam-thiazolidine ring system. Thus oxygen could be introduced into the β -position of a valine or dehydrovaline fragment by a reaction which competed with that yielding the corresponding S-C linkage of the penicillins. β -Hydroxyvaline could also be derived from an intermediate in which this S-C bond had been established. Dougall, Newton & Abraham (1957) found that the methyl ester of benzylpenicillin sulphone yielded free DL- β -hydroxyvaline on hydrolysis with 6M-hydrochloric acid at 100°C. But the formation of a β -hydroxyvaline-containing peptide by a reaction that involved the breaking of the S-C bond of an intermediate would appear unlikely unless this bond were formed before the closing of the β -lactam ring and not after it as has previously been suggested (Arnstein & Crawhall, 1957).

The result of an experiment with a mycelial suspension of the *Cephalosporium* sp. indicated that peptide P1, like peptide P3, was labelled rapidly from [14 C]valine and was consistent with an earlier finding that the amount of 14 C in the Aad-Cys-Val complex reached a maximum within 15 min (Warrén *et al.* 1967a). However, the presence of glycine in peptides P1 and P2 suggests the possibility that these peptides are derived from, but not identical with, intermediates on the main biosynthetic pathway. More results are required for a reliable assessment of the specific radioactivities of peptides P1, P2 and P3 to be made, but the present results are not inconsistent with the hypothesis that peptide P3 is a precursor of peptides P1 and P2.

The formation of peptides P2 and P3 from δ -(L- α -aminoadipyl)cysteine and DL-[14 C]valine in a broken-cell system, but not from L-cysteinyl-L-valine or L-cysteinyl-D-valine and DL- α -amino[14 C]-adipic acid, indicates that the biosynthesis of the α -aminoadipyl peptides starts from the N-terminal amino acid, as it does with glutathione (Mooz & Meister, 1967). But in the system used the biosynthesis of these peptides differed from that of glutathione in two respects. The formation of glutathione occurred almost as readily from its constituent amino acids as from γ -L-glutamyl-L-cysteine and glycine, whereas the formation of the α -aminoadipyl peptides was only demonstrated after addition of δ -(L- α -aminoadipyl)-L-cysteine. The enzymes required for the synthesis of peptides P2 and P3 were present mainly in the particulate fraction of the system, whereas those required for the synthesis of glutathione were concentrated in the supernatant. These observations indicate that the biosynthesis of the α -aminoadipyl peptides occurs at different locations in the cell from that of glutathione. The failure to detect the formation of peptide P1 in a broken-cell system may be due to

the absence from this system of a mechanism for oxidizing a valine residue to one of β -hydroxyvaline.

On the assumption that the specific radioactivity of the ^{14}C -labelled Aad-Cys-Val complex was not significantly less than that of the added L- ^{14}C -valine and that peptides P2 and P3 were not further metabolized after they had been formed, the amounts of the peptides synthesized by broken-cell preparations can be calculated. From Expt. IV of Table 2 about 15 nmol of peptide P2 and of P3 were formed in 1 h in a preparation derived from 1 g of mycelium and containing about 36 mg of protein. The amounts of extracellular penicillin N and cephalosporin C formed in this time by a suspension of 1 g of washed intact mycelium are about 180 nmol (Smith *et al.* 1967). Bauer (1970) has reported the formation of δ -(α -aminoadipyl)cysteinylvaline from its constituent amino acids in an extract of the mycelium of *P. chrysogenum*. However, in terms of the amount of protein in the extract, the amount of tripeptide formed appears to have been more than one order lower than that obtained in the present experiments.

We thank Miss Sheila Hedges for technical assistance and the National Research Development Corporation and the Medical Research Council for financial support.

REFERENCES

- Arnstein, H. R. V. & Clubb, M. E. (1957). *Biochem. J.* **68**, 528.
- Arnstein, H. R. V. & Crawhall, J. C. (1957). *Biochem. J.* **67**, 180.
- Arnstein, H. R. V. & Morris, D. (1960). *Biochem. J.* **76**, 357.
- Bauer, K. (1970). *Z. Naturf.* **25b**, 1125.
- Cole, M. & Batchelor, F. R. (1963). *Nature, Lond.*, **198**, 383.
- Dougall, D. K., Newton, G. G. F. & Abraham, E. P. (1957). *Biochem. J.* **66**, 30p.
- du Vigneaud, V. & Miller, G. L. (1952). *Biochem. Prep.* **2**, 87.
- Ellman, G. L. (1959). *Archs Biochem. Biophys.* **82**, 70.
- Flynn, E. H., McCormick, M. H., Stamper, M. C., De Valeria, H. & Godzeski, C. W. (1962). *J. Am. chem. Soc.* **84**, 4594.
- Kaplan, M. M. & Flavin, M. (1966). *J. biol. Chem.* **241**, 4463.
- Loder, P. B., Abraham, E. P. & Newton, G. G. F. (1969). *Biochem. J.* **112**, 389.
- Loder, P. B. & Abraham, E. P. (1971). *Biochem. J.* **123**, 471.
- Lowry, O. H., Rosebrough, N. J., Farr, A. I. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
- MacLaren, J. A., Savige, W. E. & Swan, J. M. (1958). *Aust. J. Chem.* **11**, 345.
- Mooz, E. D. & Meister, A. (1967). *Biochemistry, Easton*, **6**, 1722.
- Nesvadba, H. & Roth, H. (1967). *Mh. Chem.* **98**, 1432.
- Smith, B., Warren, S. C., Newton, G. G. F. & Abraham, E. P. (1967). *Biochem. J.* **103**, 877.
- Sneath, P. H. A. & Collins, J. F. (1961). *Biochem. J.* **79**, 512.
- Stevens, C. M. & Halpern, P. E. (1949). *J. biol. Chem.* **179**, 389.
- Warren, S. C., Newton, G. G. F. & Abraham, E. P. (1967a). *Biochem. J.* **103**, 891.
- Warren, S. C., Newton, G. G. F. & Abraham, E. P. (1967b). *Biochem. J.* **103**, 902.