mized rats for assay be possible, the easy replication of tests would go some way to meet this difficulty. However, the hope of an accurate assay will be somewhat dashed by (a) the presence in partly purified intrinsic-factor preparations of unknown amounts of inhibitor or inhibitors, (b) the marked dependence, in some cases, of activity on the size of the dose (e.g. 7, Table 2), (c) the possibility of quantitative differences in response of a test animal to intrinsic factor (and inhibitors) from a different animal species and (d) the possibility of a test animal becoming refractory. These points are discussed more fully by M. A. Sheikh & K. B. Taylor (unpublished work).

SUMMARY

1. Preliminary attempts to purify and examine the properties of intrinsic factor are reported.

2. Fractionation by chromatography on an exchange resin, as described by Latner, Merrills & Raine (1956) for a concentrate from pig gastric mucosa, gives closely similar results with pig pyloric secretion and human gastric juice, and appears to be a reliable and reproducible method.

3. The active fraction can be obtained several times purer by modifications of the exchange-resin process. There are reasons for thinking that the final product might be active in pernicious-anaemia patients in a dose of about 30 μ g.

4. Membrane electrophoresis suggests that the purest product is still far from homogeneous.

We are grateful to Professor Sir Howard Florey and Professor L. J. Witts for suggesting this collaboration and for their continued interest; to Mr J. Kent for tending the pig and collecting the pyloric secretion; to Miss B. Mallett for measurement of radioactivity of some of the human material; and to Mrs S. Groves for technical assistance.

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Synthesis of the α - and γ -Isomers of Glutamylcystinylvaline

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It has been shown (Arnstein & Morris, 1959, 1960b) that L-cystinyl-L-valine is available for penicillin biosynthesis in washed mycelium of *Penicillium chrysogenum* by a pathway other than that involving breakdown to free amino acids. Following this observation, the mycelium of *P. chrysogenum* was examined for the presence of cystinylvaline or of similar peptides containing both cystine and valine; at an early stage in this work there was some evidence of the presence of glutamylcystinylvaline in mycelial extracts. In order to assist in the identification of this peptide the α - and γ -isomers of glutamylcystinylvaline were synthesized.

The main problem in the synthesis of glutamyl peptides is that of producing, unequivocally, either peptides in which the α -carbonyl group is a member of the peptide chain, i.e. with part structure (A), or those in which the γ -carbonyl group participates in peptide-bond formation, as shown in part structure (B) below. Amiard, Heymès & Velluz (1956) have reported the synthesis of γ -glutamylcystinylglycine (glutathione), in which the desired γ -linkage of glutamic acid to the rest of the molecule was

obtained by the use of the triphenylmethyl (trityl) *N*-terminal-protecting group, the latter preventing by steric hindrance the participation of the α -carboxyl group in peptide-bond formation. In view of the similarity between glutathione and γ -glutamylcystinylvaline, the trityl-protecting group was used in the synthesis of this γ -peptide.

Trityl-L-glutamic acid was coupled with *S*-benzyl-L-cysteinyl-L-valine ethyl ester, prepared from the benzyloxycarbonyl-*S*-benzyl dipeptide ester (Arnstein & Morris, 1960*a*) by the action of hydrogen bromide in acetic acid, the dicyclohexylcarbodi-imide method of Sheehan & Hess (1955) being used. The fully protected tripeptide ester was saponified with ethanolic potassium hydroxide to give the trityl peptide in 77% yield. The trityl group was rather labile and was lost to a considerable extent during the purification procedure at this stage. All the trityl derivatives used in this synthesis gave ninhydrin-positive spots on paper chromatograms, the trityl group presumably being lost during the heating of the paper.

Complete removal of the trityl group was achieved by heating the protected peptide in 50% (by vol.) acetic acid at 100° for a short time. The chromatographically pure *S*-benzyl tripeptide was reduced with sodium in liquid ammonia to glutamylcystinylvaline, which was not isolated but oxidized directly to the disulphide form. Chromatography in solvent *A* (see Experimental section) revealed the presence of two components, one of which was present in only a small amount. The R_f of the minor component was the same as that of synthetic α -peptide. A portion of the mixture was oxidized with performic acid to the cysteic acid (SO_3H) form and subjected to paper electrophoresis. At pH 2 the mixture behaved as one component. At pH 3, however, where the free α -carboxyl group of the γ -peptide would be charged to a greater extent than the corresponding free γ -carboxyl group of the α -peptide, the major component migrated much faster than did the minor one. From the above-mentioned evidence it was concluded that the peptide was predominantly the required γ -isomer but contained a small proportion of the α -form. It appears therefore that steric shielding of the α -carboxyl group during peptide-bond formation was not complete. The pure γ -peptide was obtained by elution of the required band (R_f 0.12 in solvent *A*) from preparative-scale chromatograms).

The dehydration of toluene-*p*-sulphonyl (tosyl)-glutamic acid to tosylpyroglutamic acid has been known for some time (Harrington & Moggridge, 1940) but the application of this reaction to the synthesis of α -glutamyl peptides has been relatively recent. Rudinger (1954*a, b*) has reported the selective synthesis of several α -glutamyl peptides by

coupling the acid chloride derivative of tosylpyroglutamic acid with either amino acids or their esters. Accordingly, this method was used to prepare α -L-glutamyl-L-cystinyl-L-valine.

In a preliminary experiment tosyl-L-pyroglutamyl chloride (Rudinger, 1954*a*), prepared by refluxing tosyl-L-glutamic acid with thionyl chloride, was coupled with *S*-benzyl-L-cysteinyl-L-valine ethyl ester to give a very poor yield of material which could not be purified. Coupling of the *S*-benzyl dipeptide ester with tosyl-L-pyroglutamic acid by the dicyclohexylcarbodi-imide method was successful, however, the required tosyl tripeptide ester being obtained in good yield. Treatment of this product with ethanolic potassium hydroxide gave a high yield of the saponified peptide. A small portion of this material was converted into the cyclohexylamine salt; this derivative of saponified peptide intermediates has been found very useful in obtaining crystalline material from these compounds, which are otherwise often very difficult to crystallize (Arnstein & Morris, 1960*a*). Removal of the tosyl- and *S*-benzyl-protecting groups with sodium in liquid ammonia proceeded smoothly, the use of IRC-50 cation-exchange resin as described by Rudinger (1954*b*) facilitating the removal of inorganic ions from the product. Once again chromatography revealed the presence of two spots of the same R_f values as described for the γ -peptide, only with the orders of magnitude reversed. The major spot was identified as the α -peptide by electrophoresis of the $-\text{SO}_3\text{H}$ form at pH 3.5. The pure α -peptide was separated from the small amount of contaminating γ -material by crystallization from aqueous ethanol. It would appear that the pyrrolidone method of synthesis of α -glutamyl peptides is not completely specific. Rudinger & Czurbová (1954) have reported that the pyrrolidone ring can be opened with amino acid (and, presumably, peptide) esters, the reaction, however, being slow. It seems probable therefore that concomitant attack of *S*-benzyl-L-cysteinyl-L-valine ethyl ester on the pyrrolidone ring took place during the coupling reaction, giving rise to some γ -peptide.

Both α - and γ -peptides were obtained as dihydrates which could not be converted into the anhydrous forms by drying at above 100°. This tendency of peptides containing cystine and valine to form hydrates has been found with isomers of cystinylvaline (Arnstein & Morris, 1960*a*).

EXPERIMENTAL

Paper chromatography. All R_f values refer to descending chromatograms on Whatman no. 1 paper unless otherwise stated. The solvent systems used had the following compositions (by vol.): *A*, butanol-acetic acid-water, 63:10:27;

L-cysteinyl-*L*-valine ethyl ester (IV), 220 mg. (54%), m.p. 112–114°, $[\alpha]_D^{25} + 15.4^\circ$ (c, 1.04 in ethyl acetate) (Found: C, 69.8; H, 6.71; N, 6.30. $C_{11}H_{14}O_6N_2S$ requires C, 69.5; H, 6.6; N, 5.93%).

The fully protected tripeptide (IV; 1.7 g.) was dissolved in ethanolic *N*-KOH (12 ml.) and allowed to stand at room temperature for 40 min.; the solution was then brought to pH 7 with *N*-HCl and, after dilution with water, $NaHCO_3$ (2%, w/v) was added to bring the pH to 8.5. The alkaline solution was extracted with ethyl acetate (3 × 10 ml. portions) and the latter was washed with $NaHCO_3$ (2%); the combined aqueous fractions were acidified to pH 2 with conc. HCl and extracted with ethyl acetate (3 × 10 ml. portions). After drying (anhydrous Na_2SO_4) the solvent was removed to give 1.5 g. (93%) of crude product (V), m.p. 125–130°, with R_F 0.63 in solvent *B*. Several recrystallizations from ethyl acetate–light petroleum (b.p. 80–100°) gave a product, m.p. 145–150°, which could not be purified further and which was shown by chromatography in solvent *B* to contain some detritylated material (VI), R_F 0.20. The product (V, 2 g.) was suspended in water (20 ml.) at 100° and acetic acid (20 ml.) was added; starting material dissolved immediately and a product was precipitated. Water (40 ml.) was added and the reaction mixture was freeze-dried to give 1.3 g. (100%) of γ -*L*-glutamyl-*S*-benzyl-*L*-cysteinyl-*L*-valine (VI), m.p. 170–175°. This product was chromatographically pure with R_F values 0.60 in solvent *A* and 0.20 in solvent *B*; it was used without further purification.

The *S*-benzyl peptide (VI, 880 mg.) was dissolved in dry liquid NH_3 (100 ml.) contained in a three-necked flask fitted with a mechanical stirrer and a $CaCl_2$ guard tube. Sodium was introduced in small portions, with stirring, until a permanent (7 min.) blue colour was obtained; 250 mg. of sodium was required. Ammonium acetate (3 g.) was added, the NH_3 was evaporated as rapidly as possible and the residue was placed in a desiccator over conc. H_2SO_4 overnight. The dry crude product was dissolved in ice-water (100 ml.) and was stirred for 30 min. at 10° with IRC-50 in the NH_4^+ form (10 g., wet wt.; The British Drug Houses Ltd., London). The resin was filtered off and the filtrate was freeze-dried. The residue was taken up in water (20 ml.), the pH was adjusted to 8.5 with 2*N*- NH_3 soln., a trace of $FeCl_3$ was added and O_2 was bubbled through the solution [to oxidize the thiol form of the peptide (VII) to the disulphide form (VIII)] until the blue colour was discharged; the solution then gave a negative test for thiols with sodium nitroprusside. Freeze-drying of the solution gave crude product (700 mg.) which was crystallized from aqueous ethanol to give a product (600 mg., 92%) which gave two spots, R_F 0.12 (major) and R_F 0.16 (minor) in solvent *A*. The faster-moving material was later identified (see below) as α -*L*-glutamyl-*L*-cystinyl-*L*-valine. For purification the mixture (250 mg.) was chromatographed on Whatman 3MM paper (four sheets) in solvent *A* for 45 hr. The γ -peptide band (R_F 0.12) was eluted with 0.3*M*- NH_3 soln. (200 ml.) and the eluate was concentrated *in vacuo* and desalted on Zeo-Karb 215 (The Permutit Co. Ltd., London). The peptide was displaced from the column with 0.3*M*- NH_3 soln. and the eluate was concentrated *in vacuo* to 3 ml. Addition of ethanol and ether gave a solid product, which, after drying *in vacuo* for 4 hr. at 100° and then 2 hr. at 110°, gave analytically pure γ -*L*-glutamyl-*L*-cystinyl-*L*-valine dihydrate (VIII), m.p. > 300°, $[\alpha]_D^{25} - 17.7^\circ$

(c, 0.99 in 5*N*-HCl) (Found: C, 42.4; H, 6.51; N, 11.5. $C_{26}H_{44}O_{12}N_6S_2 \cdot 2H_2O$ requires C, 42.6; H, 6.6; N, 11.4%).

Synthesis of α -*L*-glutamyl-*L*-cystinyl-*L*-valine

Toluene-*p*-sulphonyl (tosyl) *L*-glutamic acid (IX, 10 g.), prepared by the method of Rudinger (1954*a*) was converted into tosyl-*L*-pyrrolid-5-one-2-carboxylic acid (X, 6.1 g., 75%), m.p. 129–130°, identical with the material described by Rudinger (1954*a*).

The above product (X, 2.65 g.) and *S*-benzyl-*L*-cysteinyl-*L*-valine ethyl ester hydrochloride (II, 3.5 g., 1 mol.prop.) were dissolved together in dry ethylene dichloride (40 ml.). Triethylamine (0.94 g., 1 mol.prop.) and dicyclohexylcarbodi-imide (2.14 g., 1.1 mol.prop.) were added to this solution; considerable evolution of heat, accompanied by deposition of dicyclohexylurea, occurred, and the reaction mixture was cooled rapidly to room temperature before being shaken mechanically for 4 hr. The urea was filtered off and the solvent was removed *in vacuo*. The residue was dissolved in $CHCl_3$ (100 ml.) and was washed successively with water, *N*-HCl, water, 50% sat. $NaHCO_3$ and water. The $CHCl_3$ was then evaporated *in vacuo* and the residue was taken up in ethyl acetate (more insoluble dicyclohexylurea was removed by filtration); slow addition of light petroleum (b.p. 80–100°) induced crystallization and, after standing overnight at –5°, the crude product (4.26 g., 75%), m.p. 92–95°, was obtained. Recrystallization of a small quantity from ethyl acetate–light petroleum (b.p. 60–80°) gave analytically pure toluene-*p*-sulphonyl-*L*-pyrrolidone-*S*-benzyl-*L*-cysteinyl-*L*-valine ethyl ester (XI), m.p. 96–100° (Found: C, 57.8; H, 6.5; N, 6.6. $C_{26}H_{37}O_7N_3S_2$ requires C, 57.7; H, 6.1; N, 6.96%). The R_F in solvent *B* was 0.93.

The fully protected peptide ester (XI, 2.01 g.) was treated with ethanolic 2*N*-KOH (8.34 ml.) for 15 min. at room temperature. The reaction mixture was then poured into 50% sat. $NaHCO_3$ (100 ml.) and extracted with ethyl acetate (3 × 20 ml.). The aqueous phase was acidified to Congo red with *N*-HCl and extracted again with ethyl acetate (3 × 20 ml.); addition of light petroleum (b.p. 60–80°) gave chromatographically pure product (1.70 g., 85%), R_F 0.61 in solvent *B*. A small quantity was recrystallized from ethyl acetate alone to give toluene-*p*-sulphonyl- α -*L*-glutamyl-*S*-benzyl-*L*-cysteinyl-*L*-valine (XII), m.p. 186–188° (Found: C, 54.5; H, 6.26; N, 6.94. $C_{27}H_{35}O_8N_3S_2$ requires C, 54.7; H, 5.9; N, 7.1%). A portion was dissolved in ethyl acetate, and cyclohexylamine and ether were added to give the tosyl peptide dicyclohexylamine salt, m.p. 180° (Found: N, 8.78. $C_{39}H_{51}O_8N_5S_2$ requires N, 8.86%).

The tosyl peptide (XII, 750 mg.) was reduced with sodium in liquid NH_3 under the same conditions as those described for the γ -peptide; 270 mg. of sodium was needed. After oxidation of the peptide from the thiol (XIII) to the disulphide (XIV) form, the aqueous solution was freed from SO_4^{2-} and SO_3^{2-} ions by the method of Rudinger (1954*b*), *m*-barium acetate being added until no further precipitate was formed. The solution was filtered, the precipitate was washed on the filter with water and the combined filtrate and washings were concentrated *in vacuo* to 30 ml. This solution was applied to a column of IRC-50 (40 g., wet wt., in the NH_4^+ form) and eluted with water until a total volume of 240 ml. was obtained; the latter

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.

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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