REFERENCES

Challinor, R. W. (1913). *J. roy. Soc. N.S.W.* **47**, 236.
Cohen, G. N., Nisman, B. & Raynaud, M. (1947). *C.R. Acad. Sci., Paris*, **225**, 647.
Cromwell, B. T. (1949). *Biochem. J.* **45**, 84.
Dessaignes, M. (1852). *Liebigs Ann.* **81**, 106.
Ducet, G. (1948). *C.R. Acad. Sci., Paris*, **227**, 871.
Dyer, F. E. & Wood, A. J. (1947). *J. Fish. Res. Bd., Canada*, **7**, 17.
Dyer, W. J. (1945). *J. Fish. Res. Bd., Canada*, **6**, 351.
Ehrlich, F. & Lange, F. (1913). *Ber. dtsch. chem. Ges.* **46**, 2746.
Feigl, F. (1946). *Qualitative Analysis by Spot Tests*, 3rd ed. New York and Amsterdam: Elsevier Publishing Co.
Guggenheim, M. (1940). *Die biogenen Amine*. Basle and New York: S. Karger.
Hanahan, D. J. & Chaikoff, I. L. (1947). *J. biol. Chem.* **169**, 699.
Hanahan, D. J. & Chaikoff, I. L. (1948). *J. biol. Chem.* **172**, 191.
Kapeller-Adler, R. & Vering, F. (1931). *Biochem. Z.* **243**, 292.
Klein, G. & Steiner, M. (1928). *Jb. wiss. Bot.* **68**, 602.
Malachowski, R. & Maslowski, M. (1928). *Ber. dtsch. chem. Ges.* **61**, 2521.
Palumbo, M. (1948). *Farm. sci e tec., Pavia*, **3**, 675. Through *Chem. Abstr.* (1949), **43**, 3145.
Richter, D., Lee, M. H. & Hill, D. (1941). *Biochem. J.* **35**, 1225.
Steele, C. C. (1949). *An Introduction to Plant Biochemistry*, 2nd ed. London: G. Bell and Sons.
Wicke, W. (1862). *Bot. Ztg*, **20**, 393.

The Synthesis of Peptides Related to Gramicidin S and the Significance of Optical Configuration in Antibiotic Peptides

2. PENTAPEPTIDES

By J. I. HARRIS and T. S. WORK
National Institute for Medical Research, Hampstead, London, N.W. 3

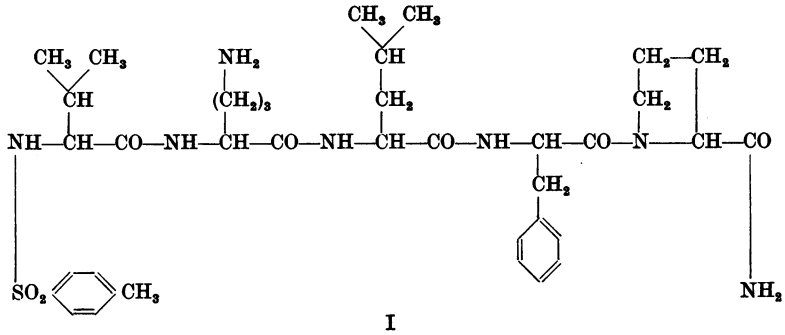
(*Received* 24 *November* 1949)

In Part 1 (Harris & Work, 1950) we reported that esters of both the tripeptides L-leucyl-L-phenylalanyl-L-proline and L-leucyl-D-phenylalanyl-L-proline synthesized by us showed some antibacterial action, but that they were very much less active than the natural antibiotic, gramicidin S, upon which they were modelled. Since there was no clear-cut distinction between the activities of the two optically isomeric peptides, it was decided to extend the peptide chain and to synthesize a pentapeptide containing all the amino-acid constituents of gramicidin S in their proper sequence and optical form, L-valyl-L-ornithyl-L-leucyl-D-phenylalanyl-L-prolyl-(II). For comparison with this peptide we synthesized the optically isomeric pentapeptide containing L-phenylalanine in place of D-phenylalanine. By comparison of the antibacterial activity of these two peptides we hoped to reveal the part played by D-phenylalanine in the production of antibiotic action.

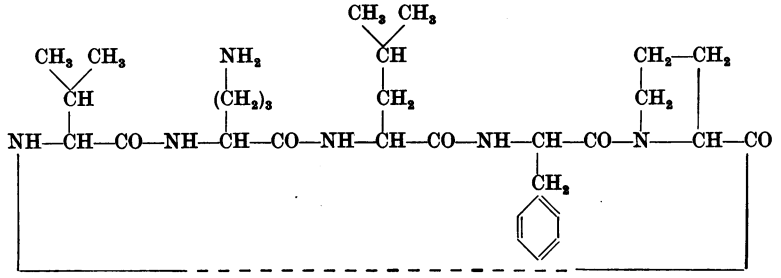
Our two isomeric open-chain pentapeptides, although comparable one with the other, are not quite comparable with gramicidin S since they possess, in addition to the free δ-amino group of the ornithine residue, a free carboxyl and a free α-amino group, both of which are absent from the natural antibiotic. The absence of reactive end groups in gramicidin S is most satisfactorily explained by the assumption of a cyclic structure. We scarcely hoped to achieve controlled cyclization of so complex a molecule as our synthetic 'D' pentapeptide and therefore approached the problem by synthesis of an acylpentapeptide amide, *α-(p-toluenesulphonyl-L-valyl-L-ornithyl-L-leucyl-D-phenylalanyl-L-proline-amide*. This compound (I) was comparable to gramicidin S (II) in that it possessed the correct amino-acid sequence, the correct optical form and the desired absence of reactive end groups.

For comparison with the acylpentapeptide possessing a free δ-NH_2 on the ornithine residue, we intended at first to prepare the corresponding pentapeptide with the α-NH_2 of valine free and the δ-NH_2 of ornithine acylated, but, as explained below, this last project was abandoned in view of unexpected experimental difficulties and because of the limited biological activity of our first acylpentapeptide amide.

The choice of the sequence valylornithylleucylphenylalanylprolyl was dictated by experimental rather than theoretical considerations. Since gramicidin S is believed to be cyclic, any one of the five constituent amino-acids can be terminal in an open-chain analogue. Preliminary experiments indicated that some of the possible intermediates would be easier to synthesize and purify than others, and the sequence finally chosen was that which seemed to



I



II

offer the best chance of complete purification and characterization of the intermediate and final products.

Synthetic procedures

The tripeptide esters, L-leucyl-D-phenylalanyl-L-proline methyl ester and L-leucyl-L-phenylalanyl-L-proline ethyl ester were already available from an earlier investigation (Harris & Work, 1950). For preliminary experiments on the lengthening of the peptide chain we used the readily obtainable DL-ornithine rather than the less accessible L-ornithine. *δ-Carbobenzyloxy-DL-ornithine* was prepared initially by the method developed by Neuberger & Sanger (1943) for δ-carbobenzyloxylysine. The yield (49 %) was disappointing. *αδ-Dicarbobenzyloxy-DL-ornithine* was obtained as a by-product. After the completion of these experiments, Synge (1948) also reported a low yield (40 %). In later experiments, using L-ornithine, we found that an excess of alkali raised the yield to 70 %; it is thus apparent that the theoretical quantity of alkali is insufficient to ensure the completion of the reaction between benzyl chloroformate and the copper complex of ornithine. δ-Carbobenzyloxy-L-ornithine methyl ester hydrochloride was prepared by the first method of Synge (1948). The pure ester hydrochloride melted at 141° (uncorr.) $[\alpha]_D^{19°} + 15{\cdot}6°$ in methanol (*c*, 3·0); Synge (1948) reported melting point 132–134°, $[\alpha]_D^{19°} + 14$–15° in methanol (*c*, 4·0). Carbobenzyloxyornithine was not coupled directly to the isomeric tripeptide esters, but was first combined with valine. L-Valine methyl ester hydrochloride prepared in this laboratory by the method of Fischer (1901) melted at 170°, whereas Synge (1948) reported melting point 146–149°.

The synthesis of α-(carbobenzyloxyvalyl)-δ-carbobenzyloxyornithine methyl ester by the azide method proved to be unexpectedly difficult. When carbobenzyloxy-L-valine azide was allowed to react at 0° with δ-carbobenzyloxy-DL-ornithine methyl ester either in ethyl acetate or in ether, the product was an intractable gel. Repeated fractionation of this gel eventually gave a low yield of the desired crystalline product. We suspected at first that our difficulty arose from the use of DL-ornithine instead of L-ornithine, but later, after we had prepared pure L-ornithine by the action of arginase on L-arginine, we were able to show that, in the reaction of carbobenzyloxy-L-valine azide with δ-carbobenzyloxy-L-ornithine methyl ester, the product was equally intractable and the yield equally bad. In the majority of peptide syntheses studied, the azide method has been the preferable method, but in this case the acid chloride method (Synge, 1948) using tosyl valine proved to be much superior.

α-(Carbobenzyloxy-L-valyl)-δ-carbobenzyloxyornithine hydrazide prepared from DL-ornithine was used for coupling with the isomeric tripeptides already synthesized (Harris & Work, 1950). It would have been desirable to use for this synthesis a dipeptide prepared from L-ornithine, but we preferred to reserve our limited supply of L-ornithine for the synthesis of the acylated pentapeptide amide which was to be compared with gramicidin S. Moreover,

we considered it unlikely that the use of DL-ornithine would invalidate our argument with regard to the significance of the optical configuration of phenylalanine. Both α-(*carbobenzyloxy*-L-*valyl*)-δ-*carbobenzyloxy*-DL-*ornithyl*-L-*leucyl*-L-*phenylalanyl*-L-*proline ethyl ester* and α-(*carbobenzyloxy*-L-*valyl*)-δ-*carbobenzyloxy*-DL-*ornithyl*-L-*leucyl*-D-*phenylalanyl*-L-*proline methyl ester* were difficult to purify and all attempts at crystallization resulted in the production of stiff gels. After repeated fractional precipitation of the amorphous compounds, correct analyses were obtained for the respective acylated pentapeptide esters and, as a check on their identity, an acid hydrolysate of each was subjected to chromatographic analysis on paper; the presence of the required amino-acids was demonstrated by comparison with an artificial mixture. Although in each case the final product gave almost theoretical analytical figures, we do not regard this as an adequate criterion of purity, and the physical constants quoted for the dicarbobenzyloxypentapeptide esters must be regarded as probable rather than absolute values.

The synthesis of an acylpentapeptide amide with a free δ-amino group on the ornithine residue required the preparation from valine and ornithine of an intermediate acyldipeptide ester with the α-NH_2 of valine and the δ-NH_2 of ornithine protected by groups of different stability. The *p*-toluenesulphonyl ('tosyl') group had a double advantage for this purpose; it was sufficiently stable to be unaffected by the conditions used for removal of a carbobenzyloxy group, and in addition it facilitated crystallization. Tosyl-L-valine was coupled with δ-carbobenzyloxy-L-ornithine methyl ester by the acid chloride method. The product, α-(*tosyl*-L-*valyl*)-δ-*carbobenzyloxy*-L-*ornithine methyl ester*, resembled α-(carbobenzyloxy-L-valyl)-δ-carbobenzyloxy-L-ornithine methyl ester in having zero rotation.

The conversion of α-(tosyl-L-valyl)-δ-carbobenzyloxy-L-ornithine methyl ester to the related hydrazide required exceptionally drastic conditions, but an excellent yield of hydrazide was eventually obtained by heating the ester under reflux with excess of 100% hydrazine hydrate in anhydrous methanol. The acylated dipeptide hydrazide was converted to the azide which coupled readily with L-leucyl-D-phenylalanyl-L-proline methyl ester to give α-(*tosyl*-L-*valyl*-δ-*carbobenzyloxy*-L-*ornithyl*-L-*leucyl*-D-*phenylalanyl*-L-*proline methyl ester*. The crude product resembled the corresponding carbobenzyloxypentapeptide ester in physical properties and tended to set to a stiff gel in all solvents. Crystallization was ultimately effected by extremely slow cooling of a saturated solution, the rate of cooling being controlled by the use of a large Dewar flask (see Experimental section). The final product was in the form of fine needles (Pl. 3*a*). Identity was checked by elementary analysis and by hydrolysis of the compound to its constituent amino-acids, which were then compared chromatographically with an artificial mixture (Pl. 3*b*).

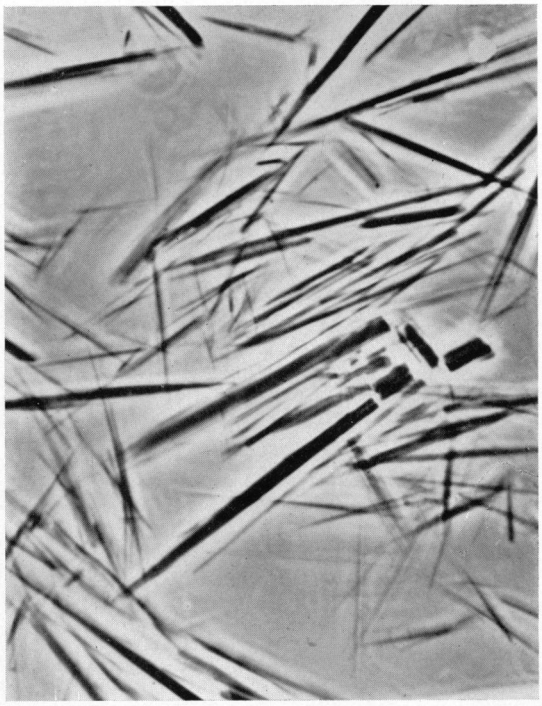
As far as can be ascertained, this is the first recorded synthesis of a crystalline derivative of a pentapeptide built from five different optically pure amino-acids. In order to complete the series of intermediates, a tetrapeptide was synthesized by the coupling of αδ-dicarbobenzyloxy-L-ornithine with L-leucyl-D-phenylalanyl-L-proline methyl ester. The azide method was used for this synthesis.

The tosyl-carbobenzyloxy-pentapeptide ester was converted to the corresponding amide by reaction with ammonia. Selective removal of the carbobenzyloxy group was achieved by catalytic hydrogeneration of the amide. Selective removal by hydrogenation of the ester was also effected. The method of removal of the tosyl group to give the free pentapeptide will be reported in a later communication.

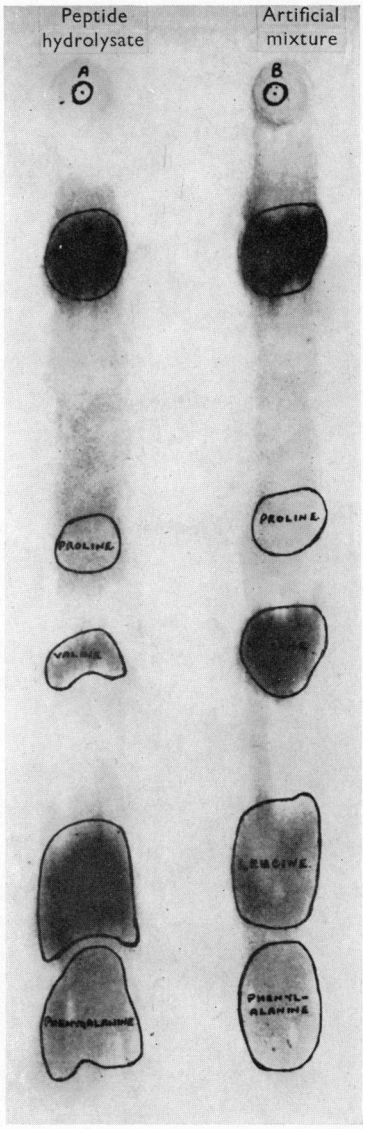
Since δ-carbobenzyloxy-L-ornithine was already available from the above synthesis we sought to couple it with phenylthiocarbonyl-L-valyl chloride. The phenylthiocarbonyl group can be removed under conditions which do not destroy the carbobenzyloxy group (Ehrensvärd, 1947), and in this way we expected to be able to synthesize a pentapeptide possessing a free α-NH_2 on the valine residue and an acyl group on the δ-NH_2 or ornithine. The addition of phenylthiocarbonyl-L-valyl chloride to two equivalents of δ-carbobenzyloxy-L-ornithine methyl ester resulted in rapid spontaneous removal of the protective phenylthiocarbonyl group. Apparently the alkalinity of the ester was sufficient to rupture the unstable thiocarbonyl linkage. *Methyl phenylthiocarbonyl*-L-*valyl*-p-*aminobenzoate* was successfully synthesized from phenylthiocarbonyl-L-valyl chloride and methyl *p*-aminobenzoate. In this case the aromatic amino group is much less basic and does not destroy the phenylthiocarbonyl group.

Antibacterial activity of products

The tetrapeptide ester, L-*ornithyl*-L-*leucyl*-D-*phenylalanyl*-L-*proline methyl ester* and the isomeric pentapeptide esters α-(L-valyl)-DL-ornithyl-L-leucyl-L-phenylalanyl-L-proline ethyl ester and α-(L-valyl)-DL-ornithyl-L-leucyl-D-phenylalanyl-L-proline methyl ester were tested for antibacterial activity, *in vitro*, against *Staphylococcus aureus*, *Streptococcus haemolyticus* and *Escherichia coli*. As can be seen from Table 1, the tetrapeptide ester and the two pentapeptide esters showed only limited activity, and the pentapeptide ester containing D-phenylalanine was not significantly more active than its isomer. The presence of the ester group in these peptides seems to have little influence on activity; thus,



(*a*)



(*b*)

(*a*) Photomicrograph by phase contrast of tosyl-L-valyl-δ-carbobenzyloxy-L-ornithyl-L-leucyl-D-phenylalanyl-L-proline methyl ester (× 1300).

(*b*) Paper-strip chromatogram of acid hydrolysate of tosyl-L-valyl-δ-carbobenzyloxy-L-ornithyl-L-leucyl-D-phenylalanyl-L-proline methyl ester (*A*) run simultaneously on Whatman no. 4 paper with an artificial mixture (*B*) of the component amino-acids; solvent system, 'collidine'-water). Ninhydrin was used for colouring the chromatogram. The yellow colour due to proline did not photograph well. Order of marked spots (from above downwards) ornithine, proline, valine, leucine, phenylalanine.

J. I. HARRIS AND T. S. WORK—THE SYNTHESIS OF PEPTIDES RELATED TO GRAMICIDIN S AND THE SIGNIFICANCE OF OPTICAL CONFIGURATION IN ANTIBIOTIC PEPTIDES. 2. PENTAPEPTIDES

Table 1. *Antibacterial activity of synthetic peptides related to gramicidin* S

(Bacteriostatic concentrations are given in the form $x \times 10^{-y}$ M; the media were as specified in Part 1 (Harris & Work, 1950).)

Substance	*Streptococcus haemolyticus* Blood	*Streptococcus haemolyticus* Broth	*Staphylococcus aureus* Broth	*Escherichia coli* Broth
L-Ornithyl-L-leucyl-D-phenylalanyl-L-proline methyl ester	—	2×10^{-3}	5×10^{-3}	4×10^{-3}
α-(L-Valyl)-DL-ornithyl-L-leucyl-D-phenylalanyl-L-proline methyl ester	$>1{\cdot}7 \times 10^{-3}$	$1{\cdot}7 \times 10^{-3}$	$>2{\cdot}0 \times 10^{-3}$	$>1{\cdot}7 \times 10^{-2}$
α-(L-Valyl)-L-ornithyl-L-leucyl-D-phenylalanyl-L-proline	—	$3{\cdot}5 \times 10^{-3}$	—	—
α-(L-Valyl)-DL-ornithyl-L-leucyl-L-phenylalanyl-L-proline ethyl ester	—	$3{\cdot}5 \times 10^{-3}$	7×10^{-3}	$>7 \times 10^{-3}$
α-(Tosyl-L-valyl)-L-ornithyl-L-leucyl-D-phenylalanyl-L-proline methyl ester	$>1{\cdot}25 \times 10^{-4}$*	$>1{\cdot}25 \times 10^{-4}$*	$>1{\cdot}25 \times 10^{-4}$*	$>1{\cdot}25 \times 10^{-4}$*
α-(Tosyl-L-valyl)-L-ornithyl-L-leucyl-D-phenylalanyl-L-proline amide	$>1 \times 10^{-4}$*	$>1 \times 10^{-4}$*	$>1 \times 10^{-4}$*	$>1 \times 10^{-4}$*
Gramicidin S	—	5×10^{-6}†	—	—

* Saturated solution. † Calculated on basis of cyclic pentapeptide.

a specimen of α-(L-valyl)-ornithyl-L-leucyl-D-phenylalanyl-L-proline prepared during our study on polymerization of pentapeptides (Harris & Work, unpublished) was found indistinguishable biologically from its ester.

α-(Tosyl-L-valyl-L-ornithyl-L-leucyl-D-phenylalanyl-L-proline methyl ester hydrochloride and α-(tosyl-L-valyl)-L-ornithyl-L-leucyl-D-phenylalanyl-L-proline amide hydrochloride were sparingly soluble in water and saturated solutions contained 10 and 8 mg./100 ml. respectively; no antibacterial activity was detected at these concentrations, whereas a sample of pure gramicidin S (kindly supplied by Dr L. C. Craig of the Rockefeller Institute, New York) was active at less than one-twentieth of these concentrations.

From the results given in Table 1 it is apparent that open-chain pentapeptides having the gramicidin S sequence of amino-acids possess only limited antibiotic action; in this respect they resemble the optically isomeric tripeptide fragments studied previously (Harris & Work, 1950). Activity does not appear to be directly related to the presence of amino-acids of 'unnatural' D-configuration since there is no appreciable difference in antibacterial potency between a pentapeptide incorporating D-phenylalanine in its structure and the corresponding peptide synthesized from L-phenylalanine.

The open chain pentapeptides differ from gramicidin S in that they possess reactive end groups (amino and carboxyl) in addition to the reactive amino group of the ornithine residue. As is evident from the formulae, synthetic α-(tosyl-L-valyl)-L-ornithyl-L-leucyl-D-phenylalanyl-L-proline amide (I) bears a close resemblance to the natural antibiotic (II); the only reactive group is the δ-amino of the ornithine residue, but the presence of this polar group in an otherwise 'closed' pentapeptide chain having the correct amino-acid sequence and optical configuration is apparently not the key to activity as was envisaged by Znamenskaya, Agatov & Belozerskiĭ (1948).

On the basis of the chemical and physical evidence so far available, gramicidin S is best formulated as a *cyclic* pentapeptide or decapeptide (Consden, Gorden, Martin & Synge, 1947; Pedersen & Synge, 1948). The experimental results presented in the present paper furnish support for the postulate that the biological aetivity of gramicidin S is intimately related to its *cyclic* structure. Further discussion of this point is reserved for a future communication.

EXPERIMENTAL

Preparative studies

Melting points are given uncorrected; optical rotations were measured in a 4 dm. tube.

*δ-Carbobenzyloxy-*DL*-ornithine.* DL-ornithine was synthesized from acrylonitrile by the acetamidomalonate method of Albertson & Archer (1945), and converted to the δ-carbobenzyloxy derivative by the method described by Neuberger & Sanger (1943) for the preparation of ε-carbobenzyloxylysine. From ornithine monohydrochloride (13 g.), *δ-carbobenzyloxy-*DL*-ornithine* (9·6 g.), m.p. 256°, was obtained in the form of needles. (Found: C, 58·3; H, 6·6; N, 10·7. $C_{13}H_{18}O_4N_2$ requires C, 58·6; H, 6·75; N, 10·5 %.) *αδ-Dicarbobenzyloxy-*DL*-ornithine* (3·2 g.), m.p. 112°, was isolated from the reaction product; this indicated incomplete stability of the Cu complex of ornithine under the conditions used. (Found: C, 63·2; H, 6·4; N, 7·3. $C_{21}H_{24}O_6N_2$ requires C, 63·0; H, 6·0; N, 7·0 %.)

*δ-Carbobenzyloxy-*DL*-ornithine methyl ester.* δ-Carbobenzyloxy-DL-ornithine (12 g.) in anhydrous methanol

(120 ml.) was saturated with dry HCl gas at room temperature, and left for 24 hr. Concentration *in vacuo* below 30° gave the ester hydrochloride as a syrup. δ-Carbobenzyloxy-DL-ornithine methyl ester hydrochloride was not crystallized. It was converted to the free ester by the usual procedure.

L-Valine methyl ester hydrochloride. L-Valine (20 g.) was converted to the ester hydrochloride by the standard procedure of Fischer (1901). L-Valine methyl ester hydrochloride crystallized from ethanol-ether, and was recrystallized from methanol-ether in the form of lustrous rectangular plates, m.p. 170°. (Found: C, 43·1; H, 8·6; N, 8·0. Calc. for $C_6H_{13}O_2N.HCl$: C, 43·0; 8·4; N, 8·4 %.)

Carbobenzyloxy-L-valine hydrazide. Carbobenzyloxy-L-valine methyl ester (Synge, 1948) (14·0 g., m.p. 56°, prepared from the ester hydrochloride) was dissolved in methanol (100 ml.) and excess hydrazine hydrate (50 % (w/v), 14 ml.) was added. The mixture, left at room temperature for 24 hr., deposited *carbobenzyloxy-L-valine hydrazide* (13·8 g.) which was recrystallized from ethyl acetate-methanol as needles (12 g.), m.p. 179°. (Found: C, 59·1; H, 7·2; N, 16·1. $C_{13}H_{19}O_3N_3$ requires C, 58·9; H, 7·1; N, 15·9 %.)

α-(Carbobenzyloxy-L-valyl-δ-carbobenzyloxyornithine methyl ester. Carbobenzyloxy-L-valine hydrazide (6 g.) in a mixture of glacial acetic acid (50 ml.) and 2N-HCl (100 ml.) was cooled to 0°, and a solution of $NaNO_2$ (1·8 g.) in water (20 ml.) was added dropwise to the stirred solution during 15 min. The acid-insoluble azide was extracted into ice-cold ethyl acetate (150 ml.) and washed successively with ice-cold water and a cold saturated solution of $NaHCO_3$ until the washings were neutral to litmus. The azide solution was dried quickly over Na_2SO_4 and added during 30 min. to a cooled solution of δ-carbobenzyloxy-DL-ornithine methyl ester (7·8 g.) in ethyl acetate (50 ml.). A gelatinous precipitate was formed almost immediately, and after 24 hr. at 0° the whole solution had set to a stiff gel; this was collected by filtration and dried at 80° to give a white amorphous solid (9 g.), m.p. 150–160°. After several precipitations from ethyl acetate a crystalline product was obtained; three recrystallizations from ethanol gave α-(carbobenzyloxy-L-valyl)-δ-carbobenzoxy-ornithine methyl ester as clusters of small needles (1 g.), m.p. 150°, $[\alpha]_D^{17°}$ +11·5° in $CHCl_3$ (*c*, 1·6). (Found: C, 62·6; H, 6·8; N, 8·3. Calc. for $C_{27}H_{35}O_7N_3$: C, 63·1; H, 6·9; N, 8·2 %.) In view of the racemic nature of the ornithine used a definite optical form cannot be assigned to this compound.

α-(Carbobenzyloxy-L-valyl)-δ-carbobenzyloxyornithine hydrazide. To a solution of the methyl ester (0·9 g.) in hot methanol (20 ml.) was added excess 90 % hydrazine hydrate (2 ml.); after 24 hr. at 37°, *α-(carbobenzyloxy-L-valyl)-δ-carbobenzyloxyornithine hydrazide* was precipitated as an amorphous gel which was collected and dried at 80° (0·75 g.). (Found: C, 60·6; H, 6·7; N, 13·9. $C_{26}H_{35}O_6N_5$ requires C, 60·8; H, 6·9; N, 13·6 %.)

α-(Carbobenzyloxy-L-valyl)-δ-carbobenzyloxyornithyl-L-leucyl-L-phenylalanyl-L-proline ethyl ester. The hydrazide (0·35 g.) was converted to the azide and extracted into ethyl acetate by the standard procedure already described; this azide was allowed to react at 0° with L-leucyl-L-phenylalanyl-L-proline ethyl ester (0·95 g.) dissolved in anhydrous ethyl acetate (50 ml.). After 24 hr. at 0° and 24 hr. at room temperature, excess tripeptide ester was extracted with N-HCl, and the ethyl acetate layer washed successively with water and saturated aqueous $NaHCO_3$. Removal of solvent under reduced pressure left a pale yellow viscous oil (0·53 g.) which was precipitated as gel, m.p. 124°, from a mixture of ethyl acetate and light petroleum. Several further precipitations from ethyl acetate-light petroleum gave a product (0·4 g.), m.p. 128°, which analysed correctly for *α-(carbobenzyloxy-L-valyl)-δ-carbobenzyloxyornithyl-L-leucyl-L-phenylalanyl-L-proline ethyl ester.* (Found: C, 65·0; H, 7·4; N, 9·8. $C_{48}H_{64}O_{10}N_6$ requires C, 65·1; H, 7·3; N, 9·5 %.) The presence of the expected amino-acids in the product was confirmed by paper chromatography of a hydrolysate.

α-(L-Valyl) ornithyl-L-leucyl-L-phenylalanyl-L-proline ethyl ester. The dicarbobenzyloxy derivative (335 mg.) was hydrogenated in methanol containing 2 equiv. HCl. *L-Valylornithyl-L-leucyl-L-phenylalanyl-L-proline ethyl ester hydrochloride* was obtained as an extremely hydroscopic semi-solid which was not crystallized.

α-(Carbobenzyloxy-L-valyl)-δ-carbobenzyloxyornithyl-L-leucyl-D-phenylalanyl-L-proline methyl ester. The method used was similar to that described above for the synthesis of the isomeric pentapeptide derivative. α-(Carbobenzyloxy-L-valyl)-δ-carbobenzyloxyornithine hydrazide (0·35 g.) was converted to the azide and coupled with L-leucyl-D-phenylalanyl-L-proline methyl ester (0·9 g.) in ethyl acetate at 0°. *α-(Carbobenzyloxy-L-valyl)-δ-carbobenzyloxyornithyl-L-leucyl-D-phenylalanyl-L-proline methyl ester* was obtained as a colourless, viscous oil which gave a white amorphous solid (400 mg.), m.p. 198–200°, from ethyl acetate-light petroleum. (Found: C, 64·5; H, 7·1; N, 10·0. $C_{47}H_{62}O_{10}N_6$ requires C, 64·8; H, 7·2; N, 9·7 %.) The presence of the expected five amino-acids was confirmed by paper chromatography after acid hydrolysis.

α-(L-Valyl) ornithyl-L-leucyl-D-phenylalanyl-L-proline methyl ester. The dicarbobenzyloxy compound was hydrogenated in methanol containing 2 equiv. HCl, and the pentapeptide ester dihydrochloride isolate as a colourless, hygroscopic syrup (160 mg.) which could not be crystallized.

L-Ornithine. L-Arginine monohydrochloride was isolated from a protein hydrolysate by the flavianic acid method of Cox (1928), and converted into L-ornithine monohydrochloride by the arginase method of Hunter (1939).

Extraction and purification of arginase. A concentrate of arginase was obtained by a modification of the method described by Bach (1946). Fresh ox liver (2·5 kg.) was minced and treated with 5 vol. (10–12 l.) acetone at room temperature. The resulting suspension was filtered, the fibrous residue thoroughly macerated with another 2 vol. (5 l.) of acetone, and filtered again. The fibrous residue was air-dried at room temperature (750 g.). This product (300 g.) was then thoroughly macerated with 0·01 N-KOH (500 ml.); a further 1500 ml. KOH solution was added and the suspension stirred for 1 hr. The resulting gelatinous suspension was filtered at 0° and the precipitate washed with 0·002 N-KOH. The combined filtrates and washings (3700 ml.) were adjusted to pH 6·8 with HCl and heated in 500 ml. quantities at 54° for 5 min., with gentle shaking. The gelatinous suspension was adjusted to pH 6·1, cooled in ice, centrifuged, and the supernatant liquid was collected (3500 ml.); this liquid was cooled to 0° and the pH readjusted to 6·8. Cold acetone (1·2 vol.; 4200 ml.) was then added with stirring and the mixture left at 0° for 30 min. Adjustment to pH 6 with a few drops of HCl facilitated flocculation of the precipitate, which was centrifuged; the gelatinous protein precipitate was redissolved in the minimum volume of distilled water, and this solution (500 ml.) was treated at 0° with 1·2 vol. (600 ml.) of cold acetone. After keeping at 0° for 1 hr. the pH was adjusted to 6·2 and the resulting enzyme precipitate

collected by centrifugation. The enzyme precipitate dissolved in glass-distilled water (160 ml.) was stored at 0°.

Arginase activity was estimated by the Warburg manometric technique using the arginase-urease method developed by Hunter & Dauphinee (1930). In this way the activity of the arginase solution prepared as above was found to be 300 Hunter units/ml., giving a yield of arginase of approximately 50,000 Hunter units from 1 kg. liver.

From L-arginine monohydrochloride (30 g.), following the procedure of Hunter (1939), L-ornithine monohydrochloride (16·2 g.), $[\alpha]_D^{18°}$ +12·02° in water (*c*, 4·0), was obtained in 70 % yield.

δ-Carbobenzyloxy-L-ornithine methyl ester. Selective δ-acylation was effected in improved yield by slight modifications of the method used by Synge (1948). L-Ornithine monohydrochloride (5 g.) was converted to the complex copper salt and treated with 1 equiv. 2N-NaOH (15 ml.) at 0°. The resulting deep blue solution was treated with 2N-NaOH (25 ml.; 1·6 equiv.) and benzyl chloroformate (4·5 ml.) added alternately in ten equal portions during 30 min. at 0°. The mixture was stirred at room temperature for another 30 min. and the pale blue precipitate collected by filtration and washed with water and ethanol. After drying, the precipitate was finely powdered, suspended in water (500 ml.) containing HCl (40 ml. 2N), stirred mechanically and decomposed by a stream of H_2S. Precipitated CuS was removed by filtration, and washed thoroughly with hot water.

δ-Carbobenzyloxy-L-ornithine (6·1 g.) was precipitated from the combined filtrate and washings by adding NaOH (40 ml. 2N), and recrystallized from 50 % (v/v) aqueous ethanol, m.p. 254°; yield 5·6 g., 70 % of theoretical.

The methyl ester hydrochloride was obtained in almost quantitative yield by the method described by Synge (1948). Thus, δ-carbobenzyloxy-L-ornithine (5 g.) gave the corresponding methyl ester hydrochloride (5·35 g.) in the form of needles, m.p. 140–141° $[\alpha]_D^{19°}$ +15·6° in methanol (*c*, 3·0).

α-(Carbobenzyloxy-L-valyl)-δ-carbobenzyloxy-L-ornithine methyl ester. Carbobenzyloxy-L-valine hydrazide (1·1 g.) was converted to the azide and allowed to react at 0° with an ethyl acetate solution of δ-carbobenzyloxy-L-ornithine methyl ester (50 % mol. excess; 1·65 g. prepared from 2 g. of the ester hydrochloride). A white gelatinous solid (0·45 g.) which precipitated from the reaction mixture was removed and the acylated dipeptide ester obtained from the filtrate by the procedure already used in the isolation of the corresponding derivative of DL-ornithine. In this way a white solid (1·2 g.) was obtained which crystallized from ethyl acetate as fluffy needles (0·8 g.), m.p. 110–116°. The product had excessive N content and the separation of pure acylated dipeptide ester from impurities proved to be very difficult. Partial purification was achieved by passing a solution of the crude substance (0·8 g.) dissolved in a 20 % (v/v) solution of $CHCl_3$ in benzene (80 ml.) through a column of 'Celite 545' (diatomaceous earth supplied by Johns Manville Co., London, S.W. 1). Crystallization of one of the eluate fractions, from ethyl acetate, gave needles (0·15 g.), m.p. 150°, $[\alpha]_D^{19°}$ ±0° in $CHCl_3$ (*c*, 3·0), which analysed correctly as α-(carbobenzyloxy-L-valyl)-δ-carbobenzyloxy-L-ornithine methyl ester (Synge, 1948). (Found: C, 63·3; H, 6·9; N, 8·4. Calc. for $C_{27}H_{35}O_7N_3$: C, 63·1; H, 6·9; N, 8·2 %.)

αδ-Dicarbobenzyloxy-L-ornithine hydrazide. αδ-Dicarbobenzyloxy-L-ornithine was prepared from L-ornithine monohydrochloride (1 g.) by the method described by Sy[illegible] (1948). The dicarbobenzyloxy derivative (1·9 g.) which crystallized from $CHCl_3$-light petroleum as fluffy needles, m.p. 114°, was converted through the methyl ester to the hydrazide by the standard procedure. *αδ-Dicarbobenzyloxy-L-ornithine hydrazide* (1·7 g.) was obtained as an amorphous solid, m.p. 126–128°. (Found: C, 60·4; H, 6·4; N, 13·6. $C_{21}H_{26}O_5N_4$ requires C, 60·9; H, 6·3; N, 13·5 %.)

αδ-Dicarbobenzyloxy-L-ornithyl-L-leucyl-D-phenylalanyl-L-proline methyl ester. αδ-Dicarbobenzyloxy-L-ornithine hydrazide (1 g.) was converted to the azide and coupled with L-leucyl-D-phenylalanyl-L-proline methyl ester (1·4 g.) in ethyl acetate solution at 0°. A small amount of a white gelatinous product (0·1 g.) insoluble in ethyl acetate, formed during the hydrazide-azide conversion, appeared to be αδ-dicarbobenzyloxy-L-ornithine amide (cf. Prelog & Wieland, 1946). The reaction *product* was isolated by the usual procedure as a viscous semi-solid which gave a pale-yellow amorphous solid (1 g.), m.p. 86–88°, on trituration with ether. (Found: C, 64·8; H, 6·7; N, 9·4. $C_{42}H_{53}O_9N_5$ requires C, 65·4; H, 6·9; N, 9·1 %.)

L-Ornithyl-L-leucyl-D-phenylalanyl-L-proline methyl ester. The dicarbobenzyloxy derivative (0·6 g.) was successfully hydrogenated in methanol containing 2 equiv. HCl. Removal of catalyst and concentration of solvent *in vacuo* gave a pale yellow, hygroscopic, amorphous solid (0·4 g.) which did not give satisfactory crystalline material. Provisional identification of the product was achieved by paper chromatography.

Phenylthiocarbonyl-L-valine. Phenylthiocarbonyl chloride was prepared by the method of Rivier (1907). Thiophenol (21 g.) gave phenylthiocarbonyl chloride (24 g.), b.p. 108°/18 mm. Hg, $d_{15°}$ 1·285.

L-Valine methyl ester (2·8 g.) was dissolved in anhydrous ether (40 ml.) and phenylthiocarbonyl chloride (0·5 equiv.; 1·85 g.) in ether (30 ml.) added, with stirring, at room temperature during 5 min. according to the general procedure of Ehrensvärd (1947). After a further 15 min. L-valine methyl ester hydrochloride (2 g.) was collected by filtration; the filtrate was washed with N-HCl and water, dried and concentrated under reduced pressure. *Phenylthiocarbonyl-L-valine methyl ester* crystallized from light petroleum in the form of needles, m.p. 60–62°. Recrystallization from *cyclo*hexanol gave clusters of needles, m.p. 64°; yield 2·3 g. (80 % of theoretical). (Found: C, 58·2; H, 6·4; N, 5·1. $C_{13}H_{17}O_3NS$ requires C, 58·4; H, 6·4; N, 5·2 %.)

The methyl ester (2·1 g.) was heated on a boiling water bath for 20 min. in a 1:1 (v/v) mixture of conc. HCl and glacial acetic acid (20 ml.). Water (40 ml.) was added to the cooled solution and *phenylthiocarbonyl-L-valine* separated as an oil which solidified on standing at 0°. The solid was crushed, washed with water and dried (1·2 g.); concentration of the combined filtrates and washings gave a second crop (0·6 g.), and recrystallization of the combined products from ethyl acetate-ligroin yielded prisms (1·6 g.), m.p. 114°. (Found: C, 56·9; H, 6·13; N, 5·5. $C_{12}H_{15}O_3NS$ requires C, 56·9; H, 6·0; N, 5·5 %.)

Phenylthiocarbonyl-L-valyl-p-aminobenzoic acid methyl ester. Phenylthiocarbonyl-L-valine (0·5 g.) and 1 equiv. (0·44 g.) PCl_5 were suspended in anhydrous ether (10 ml.), and the mixture shaken at room temperature until all the solid material had dissolved. After 1 hr. 2 vol. light petroleum were added and, on cooling the solution below 0°, phenylthiocarbonyl-L-valyl chloride crystallized as needles, (0·35 g.), m.p. 66–68° (sealed tube).

The acid chloride (0·35 g.), dissolved in anhydrous $CHCl_3$ (5 ml.), was added dropwise, with shaking, to an excess of

methyl *p*-aminobenzoate in anhydrous ether (30 ml.), and the mixture left at room temperature overnight. *p*-Aminobenzoic acid methyl ester hydrochloride was collected and the filtrate was kept gently refluxing for 3 hr.; when cool, it was washed successively with N-HCl, saturated aqueous $NaHCO_3$ and water, dried over Na_2SO_4 and concentrated *in vacuo*. The pale yellow *residue* (0·25 g.) was crystallized in the form of white needles (0·15 g.), m.p. 170°. (Found: C, 61·8; H, 5·6; N, 7·5. $C_{20}H_{22}O_4N_2S$ requires C, 62·2; H, 5·7; N, 7·2 %.)

n-p-*Toluenesulphonyl*-L-*valine* (*tosyl*-L-*valine*). To a solution of L-valine (5 g.) in N-NaOH (55 ml.) was added solid *p*-toluenesulphonyl chloride (11 g.); the mixture was stirred vigorously at room temperature for 3 hr. Excess acid chloride was removed by filtration and the filtrate acidified to congo red with dilute HCl. Tosyl-L-valine (Karrer & Veer, 1932), which separated as a white crystalline precipitate, was collected, washed with water and dried. Crystallization from a mixture of ethyl acetate-light petroleum yielded rectangular plates (6·8 g.), m.p. 144°. (Found: C, 53·4; H, 6·1; N, 5·2. Calc. for $C_{12}H_{17}O_4NS$; C, 53·1; H, 6·3; N, 5·2 %.) As there was some danger of racemization in this preparation, a sample of the product was reduced by Na in liquid NH_3 and the valine isolated. The crude valine, as isolated, had a rotation close to that of L-valine.

Tosyl-L-*valyl*-*phenylalanine methyl ester*. Tosyl-L-valine (0·6 g.) and 1 equiv. PCl_5 (0·46 g.) were suspended in anhydrous ether (10 ml.), and the mixture was shaken at room temperature until all the solid material had dissolved. After 1 hr. the ethereal solution was concentrated and 2 vol. dry light petroleum added; tosyl-L-valyl chloride crystallized on cooling the solution below 0° in the form of thin needles (0·4 g.), m.p. 63°.

A solution of this acid chloride (0·4 g.) in ether (20 ml.) was added dropwise with shaking to an ethereal solution of DL-phenylalanine methyl ester (0·7 g., 2 equiv.), and the mixture left at room temperature overnight. DL-Phenylalanine methyl ester hydrochloride (0·4 g.) was removed by filtration, and the filtrate washed free from acid, dried over Na_2SO_4 and concentrated *in vacuo*. The residue (0·4 g.) crystallized from ethyl acetate-light petroleum as white, fluffy needles of *tosyl*-L-*valyl*-*phenylalanine methyl ester* (0·3 g.), m.p. 138–139°. (Found: C, 61·2; H, 6·4; N, 6·8. $C_{22}H_{28}O_5N_2S$ requires C, 61·1; H, 6·5; N, 6·5 %.)

α-(*Tosyl*-L-*valyl*)-δ-*carbobenzyloxy*-L-*ornithine methyl ester*. An ethereal solution of tosyl-L-valyl chloride (0·8 g.) was added to a stirred and cooled solution of δ-carbobenzyloxy-L-ornithine methyl ester (1·7 g., 2 equiv.) in anhydrous ether. A white precipitate formed immediately, and the mixture was left overnight at room temperature to complete the reaction. The thick, white, crystalline precipitate was collected, washed with ether and dried (2·4 g.). Excess δ-carbobenzyloxy-L-ornithine methyl ester was recovered as the hydrochloride by washing the precipitate with water, and the water-insoluble residue (1·48 g.) was recrystallized from acetone-methanol to give the acylated dipetide ester (1·2 g.) in the form of fluffy *needles*, m.p. 187°, $[\alpha]_D^{20°} \pm 0°$ in $CHCl_3$ (*c*, 1·8). (Found: C, 58·8; H, 6·5; N, 7·9. $C_{26}H_{35}O_7N_3S$ requires C, 58·6; H, 6·6; N, 7·9 %.) In large-scale preparations by the same method the yield was 77 %, calculated on the acid chloride taken.

α-(*Tosyl*-L-*valyl*)-δ-*carbobenzyloxy*-L-*ornithine hydrazide*. The conversion of the acylated dipeptide methyl ester to the hydrazide was unusually difficult. After heating under reflux with excess of 90 % aqueous hydrazine hydrate for 2 hr. the ester was recovered almost completely unchanged. The hydrazide was, however, formed by heating the ester (2·5 g.) in anhydrous methanol (50 ml.) under reflux, with 3 equiv. A.R. hydrazine hydrate (100 %) for 6 hr. The hydrazide (2·4 g.) separated as a crystalline solid from the hot solution, and recrystallization from *iso*propanol gave pure *material*, m.p. 226–227°. (Found: C, 56·3; H, 6·6; N, 12·9. $C_{25}H_{35}O_6N_5S$ requires C, 56·3; H, 6·6; N, 13·1 %.)

α-*Tosyl*-L-*valyl*)-δ-*carbobenzyloxy*-L-*ornithyl*-L-*leucyl*-D-*phenylalanyl*-L-*proline methyl ester*. α-(Tosyl-L-valyl)-δ-carbobenzyloxy-L-ornithine hydrazide (1 g.) was converted to the azide, and a solution of the azide in dry ethyl acetate was added during 15 min. to a cooled solution of L-leucyl-D-phenylalanyl-L-proline methyl ester (1·1 g.; 50 % mol. excess) also in dry ethyl acetate (25 ml.). The solutions were well mixed and left at 0° for 24 hr. A white gelatinous precipitate (0·18 g.), m.p. 200–202°, which had separated at 0° was removed by filtration, and after 24 hr. at room temperature the filtrate deposited a fine amorphous precipitate (0·8 g.), m.p. 204–205°. Excess tripeptide ester was recovered as the hydrochloride by extracting the filtrate with N-HCl. The amorphous product, m.p. 204–205°, was crystallized from methanol by a special technique. The substance was heated under reflux with methanol, filtered hot to remove a small amount of sparingly soluble impurity, and the filtrate left to cool slowly by immersing the securely stoppered containing flask in water at 50° in a large Dewar flask. This procedure ensured a slow steady rate of cooling over a period of 3 days, and in this way α-(*tosyl*-L-*valyl*)-δ-*carbobenzyloxy*-L-*ornithyl*-L-*leucyl*-D-*phenylalanyl*-L-*proline methyl ester* crystallized as ball-shaped clusters of fine microneedles (3·6 g.), m.p. 200° (Pl. 3*a*). (Found: C, 62·0; H, 7·0; N, 9·3; S, 3·9. $C_{46}H_{62}O_{10}N_6S$ requires C, 62·0; H, 7·0; N, 9·4; S, 3·6 %.)

A second preparation from α-(tosyl-L-valyl)-δ-carbobenzyloxy-L-ornithine hydrazide (1·14 g.) gave the acylated pentapeptide ester (1·4 g.), crystallized by the procedure described above, as aggregates of microneedles, m.p. 201°; identity of the product was confirmed by paper chromatography of a hydrolysate (Pl. 3*b*).

α-(*Tosyl*-L-*valyl*)-L-*ornithyl*-L-*leucyl*-D-*phenylalanyl*-L-*proline methyl ester monohydrochloride*. α-(Tosyl-L-valyl)-δ-carbobenzyloxy-L-ornithyl-L-leucyl-D-phenylalanyl-L-proline methyl ester (0·5 g.) was dissolved in methanol (100 ml.) containing 1 equiv. HCl. Hydrogenation was effected in the presence of Pd black and evolution of CO_2 was complete in 90 min. After removal of catalyst the filtrate was concentrated *in vacuo*; the oily residue was dissolved in methanol and crystallized by the cautious addition of ether, yielding *microneedles* (0·42 g.), m.p. 236° (decomp.), $[\alpha]_D^{18°} -94·6°$ in methanol (*c*, 1·0). (Found: C, 57·4; H, 7·36; N, 10·7; Cl, 4·3. $C_{38}H_{56}O_8N_6S$.HCl requires C, 57·5; H, 7·2; N, 10·6; Cl, 4·5 %.)

α-(*Tosyl*-L-*valyl*)-L-*ornithyl*-L-*leucyl*-D-*phenylalanyl*-L-*proline amide monohydrochloride*. The diacylpentapeptide methyl ester (0·25 g.) was dissolved in methanol previously saturated with anhydrous NH_3 at 0° (10 ml.), and the solution kept at 37° in a sealed tube for 72 hr. The solvent and excess NH_3 were removed *in vacuo*; the solid residue was redissolved in methanol containing 1 equiv. HCl, and hydrogenated in the presence of Pd black by the usual method. After removal of catalyst, the filtrate was concentrated and adjusted to pH 4; the amide hydrochloride crystallized in the form of fine needles, m.p. 256–260° (decomp.), on the

cautious addition of ether. Recrystallization from methanol-ether gave *microneedles*, m.p. 268–270° (decomp.). (Found: N, 13·0%. $C_{37}H_{55}O_7N_7S.HCl$ requires N, 12·6%.)

SUMMARY

1. Two optically isomeric open-chain pentapeptide esters have been synthesized. These peptides have the same sequence of amino-acid residues as gramicidin S.

2. A crystalline acylpentapeptide amide has been synthesized. This compound is structurally analogous to the natural antibiotic in that its constituent amino-acids are arranged in the same sequence and are of the same optical configuration as those of gramicidin S and that its only reactive group is the δ-NH_2 of the ornithine residue.

3. These and other newly synthesized peptides were tested, *in vitro*, for antibacterial activity.

4. The bearing of the results on the question of the origin of antibiotic activity in gramicidin S is discussed.

We are indebted to Dr A. T. Fuller for the biological tests summarized in Table 1, and to Mr J. Smiles for the photograph of crystalline acylpentapeptide ester.

REFERENCES

Albertson, N. F. & Archer, S. (1945). *J. Amer. chem. Soc.* **67**, 2043.
Bach, S. J. (1946). *Nature, Lond.*, **158**, 376.
Consden, R., Gordon, A. H., Martin, A. J. P. & Synge, R. L.M. (1947). *Biochem. J.* **41**, 596.
Cox, G. J. (1928). *J. biol. Chem.* **78**, 475.
Ehrensvärd, G. C. H. (1947). *Nature, Lond.*, **159**, 500.
Fischer, E. (1901). *Ber. dtsch. chem. Ges.* **34**, 433.
Harris, J. I. & Work, T. S. (1950). *Biochem. J.* **46**, 196.
Hunter, A. (1939). *Biochem. J.* **33**, 27.
Hunter, A. & Dauphinee, J. A. (1930). *J. biol. Chem.* **85**, 627.
Karrer, P. & Veer F. C. van der S. (1932). *Helv. chim. Acta*, **15**, 746.
Neuberger, A. & Sanger, F. (1943). *Biochem. J.* **37**, 515.
Pedersen, K. O. & Synge, R. L. M. (1948). *Acta chem. Scand.* **2**, 408.
Prelog, V. & Wieland, P. (1946). *Helv. chim. Acta*, **29**, 1128.
Rivier, M. H. (1907). *Bull. Soc. chim.* (4) **1**, 733.
Synge, R. L. M. (1948). *Biochem. J.* **42**, 99.
Znamenskaya, M. P., Agatov, P. A. & Belozerskiĭ, A. N. (1948). *Doklady Akad. Nauk S.S.S.R.* **59**, 95. Through *Chem. Abstr.* **42**, 6402 (1948).

Proteolytic Enzymes of *Clostridium welchii*

By ETHEL BIDWELL*
The Wellcome Research Laboratories, Beckenham, Kent

(*Received* 2 *December* 1949)

Culture filtrates from *Clostridium welchii* Type A contain an enzyme, collagenase (called κ-toxin by Oakley, Warrack & van Heyningen, 1946), which dissolves native collagen, softens muscle and also attacks degraded collagen as in hide powder and 'azocoll' (hide powder coupled with a red dye). The partial purification and some biochemical properties of this enzyme were described by Bidwell & van Heyningen (1948) and by Bidwell (1949*a*); observations on its effect *in vivo* and its action on gelatin were made by Oakley, Warrack & Warren (1948).

Walbum & Reymann (1934) found that filtrates from a strain of the lamb dysentery bacillus (*Cl. welchii* Type B) liquefied gelatin optimally at pH 7. Oakley *et al.* (1948) showed that *Cl. welchii* Type B produced a substance which attacked hide powder, azocoll and gelatin, but appeared to be without effect on undegraded collagen and muscle; it was antigenic and was designated the λ-antigen of *Cl. welchii*. It was inhibited by a substance in normal horse serum which is not identical with the trypsin inhibitor (Bidwell, 1949*b*). The present paper shows that the λ-antigen of Oakley *et al.* (1948) has the properties of a proteolytic enzyme; it will be referred to as 'λ-enzyme'. Its partial purification and properties will be described and compared with those of collagenase and the 'secondary' enzyme formed by the treatment of collagenase preparations with mild alkali or heat (Bidwell, 1949*a*).

* Now at Radcliffe Infirmary, Oxford.

METHODS

Estimation of enzymic activity. The ability of λ-enzyme preparations to disintegrate azocoll was determined in '*R* units' in terms of a standard dried preparation by measuring the amount of dye liberated from azocoll under standard conditions. The standard preparation was crude culture filtrate of *Cl. welchii* Type B grown on meat broth medium containing 1% Parke Davis peptone, which was preserved by drying from the frozen state. The standard preparation contained 0·2*R* unit of λ-enzyme/mg. by definition. The