407. *Studies on Specific Chemical Fission of Peptide Links. Part II.* The Preparation and Hydrolysis of 1-Acylpyrrolid-2-ones Derived from α-Glutamyl Peptides.*

By Alan R. Battersby and J. C. Robinson.

α-Esters and α-dialkylamides of *N*-acylglutamic acids have been cyclised by thionyl chloride at 0° to the corresponding 1-acylpyrrolid-2-ones of types (IX) and (II) respectively. Under similar conditions, the α-glutamyl peptide (I*g*) is cyclised in high yield to the acylpyrrolidone (II*g*), whereas cyclisation by way of the mixed carbonic anhydride gave the imide (VI*g*). An examination of the hydrolysis of all these cyclic products with cold dilute alkali has shown that a useful amount of specific fission of the peptide chain at the nitrogen atom of the glutamyl residue can be achieved by way of acylpyrrolidones. However, when imide formation is possible, rearrangement of part of the acylpyrrolidone by alkali to the imide is a complicating feature of the hydrolysis.

Part I* of this series described the first step of a scheme for specific fission of peptide chains at those points where aspartic and glutamic acid residues occur. Though this route still occupies our attention, we also wished to develop shorter routes. One such scheme for fission of a peptide chain adjacent to glutamic acid residues included the conversion of α-glutamyl peptides (I) into 1-acylpyrrolid-2-ones (II). Being diacylamines, these should be readily hydrolysed by dilute alkali.[1] Moreover, in the absence of powerful steric hindrance, specific fission [at (*a*) in II] would be expected to be favoured over ring-opening [at (*b*) in II], since the former is a result of attack by hydroxyl ion at the more electrophilic carbonyl group.[2,3] However, the desired cyclisation of the peptides (I) to acylpyrrolidones (II) is subject to competition from the alternative ring-closure leading to imides (VI). Indeed, when Clayton and Kenner[4] treated an acylated α-glutamyl tetrapeptide with thionyl chloride and pyridine, only the related imide was isolated. Cyclisation of α-glutamyl peptides to imides has also been realised by a different method in this laboratory[3] and by Sondheimer and Holley.[5]

No examples occur in the literature of cyclisation of a peptide of type (I) to an acylpyrrolidone (II), though in two cases[6,7] α-benzyl esters of *N*-acylglutamic acid (VIII; R′ = CH_2Ph, R″ = H) have been converted into acylpyrrolidones (IX; R′ = CH_2Ph). Here, however, only acylpyrrolidone formation is possible, and the products were not further examined. Accordingly, we undertook a study of the preparation and hydrolysis of several 1-acylpyrrolid-2-ones, in cases without and in cases with competition from imide formation.

α-Ethyl *N*-benzoyl-DL-glutamate (VIII*a*) was cyclised at 0° by a large excess of thionyl chloride, which also acted as solvent, to give 84% of 1-benzoyl-5-ethoxycarbonyl-DL-pyrrolid-2-one (IX*a*). This is a stable compound under neutral or slightly acidic conditions and is unaffected by a short treatment with aqueous sodium hydrogen carbonate. It is so rapidly attacked by aqueous alkali, however, that the reaction can be run as a slow titration. When the hydrolysis was conducted in ethanol at room temperature with 1 equiv. of 0·1N-sodium hydroxide, the solvent participated in the reaction and 26% of diethyl *N*-benzoyl-DL-glutamate[8] (VIII*b*), formed by ring-opening, was isolated in addition to 13% of benzoic acid formed by fission of the chain. It seemed probable, in view of the isolation of the diester (VIII*b*), that ethyl benzoate is also a reaction product and that, in

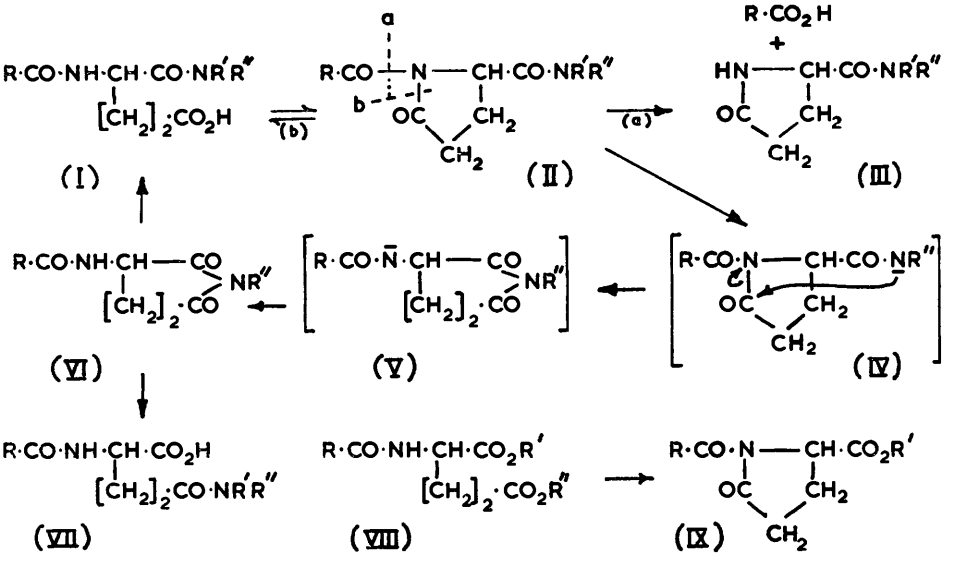
* Part I, *J.*, 1955, 259.

[1] Titherley and Stubbs, *J.*, 1914, **105**, 299.
[2] Emery and Gold, *J.*, 1950, 1455.
[3] Battersby and Robinson, *J.*, 1955, 259.
[4] Clayton and Kenner, *Chem. and Ind.*, 1953, 1205.
[5] Sondheimer and Holley, *J. Amer. Chem. Soc.*, 1954, **76**, 2467.
[6] Berenborn and White, *ibid.*, 1949, **71**, 2246.
[7] King and Spensley, *J.*, 1950, 3159.
[8] Abderhalden and Rossner, *Z. physiol. Chem.*, 1926, **152**, 271.

consequence, the yield of benzoic acid above is not a true measure of specific fission. The hydrolysis was, therefore, repeated in aqueous dioxan and a 42% yield of benzoic acid was obtained, representing fission of 42% of the acylpyrrolidone (IX*a*) in the desired manner.

The α-ethyl *N*-benzoyl-DL-glutamate (VIII*a*) used in the foregoing experiments was prepared, in admixture with the γ-ethyl isomer and diethyl *N*-benzoyl-DL-glutamate (VIII*b*), by heating *N*-benzoyl-DL-glutamic anhydride [3] with ethanol. The acidic products were separated by countercurrent distribution and orientated by identification of the stronger acid as γ-ethyl *N*-benzoyl-DL-glutamate (VIII*c*) of unequivocal structure.[3] Also, the conversion of the weaker acid into the acylpyrrolidone (IX*a*) described above supports the assigned structures. The α-ester made up roughly 80% of the acidic products, in keeping with earlier experience of ring-openings involving *N*-benzoyl-aspartic and -glutamic anhydride.[9, 10, 3]

In order to study an acylpyrrolidone derivative having the acyl group derived from an *N*-acylamino-acid, the peptide *N*-benzoylglycyl-DL-glutamic acid α-diethylamide (I*d*) was prepared. This involved coupling *N*-benzoylglycine with dimethyl glutamate [11] by the mixed anhydride method [12] and hydrolysing the resultant diester (VIII*e*) with alkali to give *N*-benzoylglycyl-DL-glutamic acid (VIII*f*). Condensation of this with diethylamine



Series (*a*): R = Ph, R′ = Et, R″ = H.
Series (*b*): R = Ph, R′ = R″ = Et.
Series (*c*): R = Ph, R′ = H; R″ = Et.
Series (*d*): R = Ph·CO·NH·CH_2, R′ = R″ = Et.
Series (*e*): R = Ph·CO·NH·CH_2, R′ = R″ = Me.
Series (*f*): R = Ph·CO·NH·CH_2, R′ = R″ = H.
Series (*g*): R = Ph; R′ = H, R″ = CH_2·CO·NH·$[CH_2]_5$·Me.
Series (*h*): R = Ph, R′ = CH_2Ph, R″ = H.
Series (*i*): R = Ph, R′ = H, R″ = CH_2Ph.
Series (*j*): R = Ph, R′ = R″ = CH_2Ph.

by the mixed anhydride method, with only *one* equivalent each of ethyl chloroformate and triethylamine, afforded a mixture of the isomeric α- and γ-glutamyl peptides, (I*d*) and (VII*d*) respectively, which were readily separated by virtue of their different acidic strengths. The weaker acid was assigned the former structure, an assignment which was confirmed by reactions carried out subsequently on this acid.

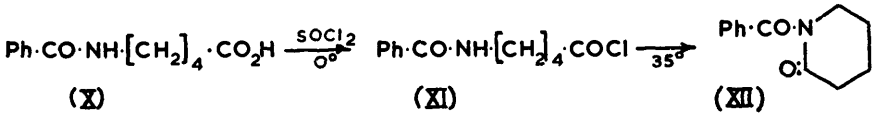
The last step in the above synthesis appears to offer some advantages over methods involving protection of one carboxyl group for those preparations of glutamyl peptides in which simplicity is more important than a high yield of one isomer. In the case examined,

[9] Pauly and Weir, *Ber.*, 1910, **43**, 661.
[10] King and Kidd, *J.*, 1951, 2976.
[11] Hillmann, *Z. Naturforsch.*, 1946, **1**, 682.
[12] Boissonnas, *Helv. Chim. Acta*, 1951, **34**, 874; Vaughan, *J. Amer. Chem. Soc.*, 1951, **73**, 3547; Wieland and Bernhard, *Annalen*, 1951, **572**, 190.

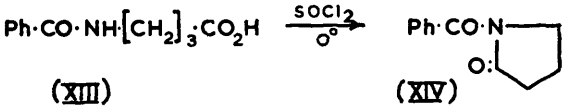
the combined yield of the α- and γ-glutamyl peptides, before fractionation was 70%. The separation of such mixtures of peptides is possible quantitatively by countercurrent distribution or, less efficiently but more simply, by fractional precipitation.

When the foregoing *N*-benzoylglycyl-DL-glutamic acid α-diethylamide (I*d*) was cyclised with thionyl chloride at 0°, a 90% yield of the 1-acylpyrrolid-2-one (II*d*) was obtained. This was hydrolysed rapidly at room temperature by 0·1N-sodium hydroxide in aqueous dioxan and the acidic products were separated by fractional precipitation with acid from a solution of their sodium salts. The weaker acid (23%) was recovered starting material (I*d*) formed by opening the pyrrolidone ring [(*b*) in II] and the stronger acid (36%) was *N*-benzoylglycine arising by fission of the peptide chain [(*a*) in II]. The amount of chain fission is probably higher than 36% since the quoted yields of the two acids represent isolated solids and account for only 59% of the acylpyrrolidone hydrolysed.

Finally, a study was made of the cyclisation of *N*-benzoyl-α-DL-glutamylglycine *n*-hexylamide [3] (I*g*) where acylpyrrolidone and imide formation are possible. An indication of the conditions which might favour the former cyclisation over the latter came from Kanewskaja's experiments.[13] This worker found that δ-benzamidovaleric acid (X) with thionyl chloride at 0° yielded the corresponding acid chloride (XI) which was moderately



(X) (XI) (XII)



(XIII) (XIV)

stable at 0°, but cyclised to 1-benzoylpiperid-2-one (XII) at 35°. The acid chloride from γ-benzamidobutyric acid (XIII), however, cyclised even at 0° to 1-benzoylpyrrolid-2-one (XIV). Accordingly, the peptide (I*g*) was treated with an excess of thionyl chloride at 0° for 10 hr., 94% of a crystalline neutral product *A* being obtained. Neither longer treatment times nor treatment at −20° for 19 hr. appreciably affected the yield, but cyclisation was incomplete after 2 hr. at 0°, only 34% of *A* being isolated. Analysis showed that *A* had been formed from the peptide (I*g*) by loss of one molecule of water.

When this product *A* was treated with 1 equiv. of 0·1N-sodium hydroxide in aqueous dioxan, ready hydrolysis occurred and benzoic acid was isolated in 45% yield. The neutral hydrolysis product, isolated in 45% yield, is presumably 5-oxo-DL-pyrrolidine-2-carbonylglycine *n*-hexylamide (III*g*), but we were unable to isolate it analytically pure. The remaining products were acidic and were shown by countercurrent distribution to consist of a mixture of 54% of the α-glutamyl peptide (I*g*) and 46% of the γ-glutamyl isomer [3] (VII*g*). Together, these two acidic peptides account for 44% of the original acylpyrrolidone.

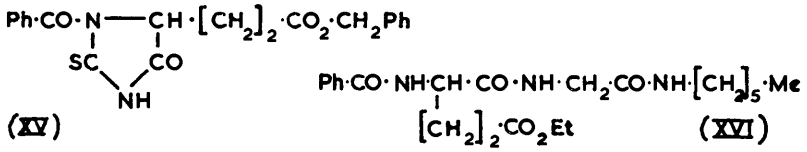
Isolation of the γ-glutamyl peptide (VII*g*) from the hydrolysis products of *A* shows either that *A* is a mixture of the required acylpyrrolidone (II*g*) and the imide (VI*g*) or that *A* is one of the pure isomers (II*g*) and (VI*g*) which undergoes rearrangement during alkaline hydrolysis. Against the first possibility it was found that *A* was unchanged by repeated recrystallisation and also by countercurrent distribution for 1060 transfers. Moreover, the distribution curve had the theoretical shape. Evidence in favour of the second possibility was therefore sought by synthesis of the acylpyrrolidone derivative (II*g*) in the following way.

α-Benzyl *N*-benzoyl-DL-glutamate (VIII*h*) was prepared and separated from the accompanying γ-benzyl isomer (VIII*i*) and dibenzyl *N*-benzoyl-DL-glutamate (VIII*j*) as for the analogous ethyl esters above. The assignments of structure to the monobenzyl esters on the basis of acidic strength were confirmed by conversion of the stronger acid (γ-benzyl

[13] Kanewskaja, *Ber.*, 1936, **69**, 266.

ester) into the thiohydantoin (XV) with acetic anhydride and ammonium thiocyanate, a reaction characteristic of γ-esters and γ-amides in the *N*-acylglutamic acid series.[14] These reagents left the weaker acid (α-benzyl ester) largely unchanged. The α-benzyl ester (VIII*h*) was then cyclised with thionyl chloride at 0° to give 93% of 5-benzyloxycarbonyl-1-benzoyl-DL-pyrrolid-2-one (IX*h*) which was smoothly hydrogenolysed over palladium–charcoal. The resultant 1-benzoyl-5-oxo-DL-pyrrolidine-2-carboxylic acid (IX*i*) was converted into the mixed carbonic anhydride (IX; R = Ph, R′ = CO_2Et) which was treated slowly with glycine *n*-hexylamide.[3] These conditions allowed preferential attack by the base at the more reactive anhydride function rather than on the diacylamine grouping. The neutral product (II*g*) was identical with product *A* and, as would be expected, gave on hydrolysis with alkali the same products in the same amounts, within experimental error, as did *A*. The hydrolysis products were benzoic acid (51%), a neutral fraction (50%), and an acidic mixture consisting of 55% of the α-glutamyl peptide (I*g*) and 45% of the γ-glutamyl peptide (VII*g*).

Further evidence in support of the acylpyrrolidone structure (II*g*) for *A* and for *A*'s homogeneity was forthcoming when the α-glutamyl peptide (I*g*) was converted into its mixed carbonic anhydride (I*g*; $CO{\cdot}O{\cdot}CO_2Et$ in place of CO_2H) and this was allowed to cyclise at low temperature. The neutral product was isomeric with *A*, but differed sufficiently from it to admit of their ready separation, after mixing, by fractional crystallisation. This second product is clearly the imide (VI*g*) since it underwent ready hydrolysis by 0·1N-sodium hydroxide to give a mixture consisting of 78% of the γ-glutamyl peptide (VII*g*) and 22% of the α-glutamyl peptide (I*g*). These accounted for 93% of the imide hydrolysed and no benzoic acid could be detected in the hydrolysate. This result is of importance for our main argument, but it also allows an answer to be given to a problem which was left open in Part I of this series.[3] There the ester (XVI) was rearranged



by aqueous sodium hydroxide to give a mixture of the α- and γ-glutamyl peptides (I*g*; 43%) and (VII*g*; 57%) respectively, the rearrangement proceeding by way of the imide (VI*g*). At that time the extent to which simple ester hydrolysis contributed to the yield of the α-glutamyl peptide (I*g*) was not known, but comparison of the relative amounts of the peptides obtained from the ester (XVI) and from the imide (VI*g*) shows that ester hydrolysis competes significantly with the rearrangement in contrast to the aspartyl analogue.[3]

The foregoing results show that the α-glutamyl peptide (I*g*) can by cyclised in high yield to the required acylpyrrolidone (II*g*) and that when this is treated with dilute sodium hydroxide, three competing reactions occur: (1) in part, the peptide chain is cleaved at (*a*) in (II*g*); (2) part undergoes ring-opening at (*b*) in (II*g*); (3) the rest rearranges, presumably by way of the anions (IV*g*) and (V*g*), to the imide (VI*g*) which is hydrolysed further to a mixture of the α- and γ-glutamyl peptides (I*g*) and (VII*g*), respectively. Knowing the proportions of (I*g*) and (VII*g*) produced by hydrolysis of the pure imide (VI*g*) it is possible to make a rough estimate of the relative importance of the three reactions above. About 45% of the acylpyrrolidone (II*g*) undergoes reaction (1), 25% reaction (2), and 30% reaction (3).

The fact that no benzoic acid could be detected when the imide (VI*g*) was hydrolysed shows that the reverse rearrangement from imide to acylpyrrolidone (II*g*) occurs at most to a very small extent. Thus, in this case, the imide is the more stable isomer and the initial cyclisation of the α-glutamyl peptide (I*g*) to the acylpyrrolidone must be subject to kinetic and not thermodynamic control.

The outcome of our work so far is that a useful amount of fission of the peptide chain

[14] Swan, *Austral. J. Sci. Res.*, 1952, *A*, **5**, 721.

has been achieved under mild conditions, though the occurrence of rearrangement (not in *order* of amino-acid residues, however) will make separation of the fission products more difficult when more than two glutamyl residues occur in the oligopeptide under study. But recent work, *e.g.*, on insulin,[15] tyrocidine A,[16] and oxytocin [17] has shown that modern techniques can deal successfully with mixtures of peptides more complex than would be expected to arise from chain fission by way of acylpyrrolidones. Our method is therefore to be tested on a variety of more complex peptides for a fuller evaluation.

Experimental

Analyses are by Mr. B. S. Noyes and his colleagues (Bristol). Analytical samples were dried at 100° *in vacuo* over phosphoric oxide unless otherwise stated. Evaporations were carried out at 40° under reduced pressure. Congo-red was the indicator used for acidification unless otherwise stated.

α-Ethyl N-*Benzoyl*-DL-*glutamate* (VIII*a*).—A suspension of *N*-benzoyl-DL-glutamic anhydride [3] (3 g.) in absolute ethanol (60 ml.) was heated under reflux for 3 hr. The gum, recovered by evaporation of the solution, was dissolved in ether (20 ml.), and the solution was shaken with 0·25N-sodium carbonate (3 × 11 ml., finally with 25 ml.). The ether was dried and evaporated and the residue (0·19 g.) was crystallised from light petroleum and then from aqueous ethanol, to give diethyl *N*-benzoyl-DL-glutamate, m. p. 76—79°. Abderhalden and Rossner [8] record m. p. 77—78°.

The fourth alkaline extract above, when acidified, precipitated a crystalline solid (0·302 g.), m. p. 97·5—99·5°, shown to be *N*-benzoyl-DL-glutamic acid monohydrate by comparison with an authentic specimen. Acidification of the first, second, and third extracts gave oils which were extracted together into ethyl acetate and recovered as a clear gum (2·78 g.) by evaporation. This was fractionated by countercurrent distribution (scattered in the first 5 tubes; 37 transfers) between ethyl acetate and aqueous phosphate buffer made from 0·5M-KH_2PO_4 (11 vol.) and 0·5M-K_2HPO_4 (1·6 vol.). The solutions from tubes 20—40 were combined, and the separated aqueous layer was acidified and extracted with ethyl acetate. After being washed with water, the upper layer from these tubes, and the extracts were dried and evaporated to give *α-ethyl* N-*benzoyl*-DL-*glutamate* as a gum (1·81 g., 50%). It could not be induced to crystallise and was purified for analysis by short-path distillation at 150—160° (bath)/0·01 mm. (Found: C, 59·9; H, 6·2; N, 5·0. $C_{14}H_{17}O_5N$ requires C, 60·2; H, 6·1; N, 5·3%). The solute from tubes 2—18 was isolated in the same way as a gum (0·45 g., 12%) which crystallised from aqueous ethanol to give *γ*-ethyl *N*-benzoyl-DL-glutamate, m. p. 110—112°, raised to 112—114° in admixture with an authentic sample [3] of m. p. 115—115·5°.

5-*Ethoxycarbonyl*-1-*benzoyl*-DL-*pyrrolid*-2-*one* (IX*a*).—α-Ethyl *N*-benzoyl-DL-glutamate (0·28 g.) was dissolved in purified thionyl chloride (1 ml., 14 equiv.) at 0° and the solution was kept at 0° for 15 hr. Evaporation of the thionyl chloride at 0° left an oil which was partitioned between ether (7 ml.) and 3% aqueous sodium hydrogen carbonate. The ethereal layer was washed with water, dried, and evaporated to leave a colourless gum (0·218 g., 84%). Crystallisation from ether–light petroleum (b. p. 60—80°) gave 1-*benzoyl*-5-*ethoxycarbonyl*-DL-*pyrrolid*-2-*one* as plates (0·148 g.), m. p. 54—58°, raised to 55·5—58° by repeated crystallisation from aqueous ethanol (Found, in material dried at 35°: C, 64·8; H, 5·6; N, 5·6. $C_{14}H_{15}O_4N$ requires C, 64·4; H, 5·8; N, 5·4%).

Alkaline Hydrolysis of 1-*Benzoyl*-5-*cthoxycarbonyl*-DL-*pyrrolid*-2-*one* (IX*a*).—(a) *In ethanol.* 0·1N-sodium hydroxide (11·3 ml., 1 equiv.) was added gradually to a solution of the acylpyrrolidone (IX*a*) (0·292 g.) in ethanol (8 ml.). About 0·5 equiv. of alkali was consumed rapidly (12 min.) and further consumption was slow. After evaporation of the alcohol, the oily suspension was extracted with ether and the extract washed with water, dried, and evaporated to a gum. This gave, on crystallisation from aqueous ethanol, diethyl *N*-benzoyl-DL-glutamate (92 mg., 26%), m. p. and mixed m. p. 77—79°.

The aqueous alkaline solution above was concentrated to 7 ml., then acidified, and the precipitated benzoic acid was recrystallised from water (18 mg., 13%), m. p. and mixed m. p. 120—121°.

(b) *In aqueous dioxan.* A solution of the acylpyrrolidone (IX*a*) (20 mg.) in dioxan (0·5

[15] Sanger and Tuppy, *Biochem. J.*, 1951, **49**, 463, 481; Sanger and Thompson, *ibid.*, 1953, **53**, 353, 366.
[16] Paladini and Craig, *J. Amer. Chem. Soc.*, 1954, **76**, 688.
[17] Du Vigneaud, Ressler, and Trippett, *J. Biol. Chem.*, 1953, **205**, 949; Tuppy, *Biochim. Biophys. Acta*, 1953, **11**, 449.

ml.) and water (1 ml.) was titrated with 0·1N-sodium hydroxide (0·8 ml., 1 equiv.); all the alkali was consumed in 10 min. The dioxan was evaporated, the aqueous suspension extracted with ethyl acetate (20 ml.), and the latter washed with water. After concentration of the combined aqueous solutions, they were acidified and extracted thrice with light petroleum (b. p. 40—60°; total 90 ml.). The petroleum extracts were combined, dried, and evaporated, to leave benzoic acid (3·9 mg., 42%), m. p. 115—121°, raised to 118—121° in admixture with a pure sample. This yield of benzoic acid and subsequent ones have been corrected as a result of trial recovery experiments by the above method.

Dibenzyl N-*Benzoyl*-DL-*glutamate* (VIII*j*) *and* α-*Benzyl* N-*Benzoyl*-DL-*glutamate* (VIII*h*).—*N*-Benzoyl-DL-glutamic anhydride (5·08 g.) and benzyl alcohol (15 ml.) were heated together for 4 hr. at 110°. The solution was evaporated to dryness at 150° (bath)/1 mm. and the residue crystallised from ether (35 ml.), to give prisms (3·02 g.), m. p. 126—127°, after sintering. This crop, in ethyl acetate (100 ml.), was shaken with an excess of 0·5N-sodium carbonate (total 40 ml.), and the ethyl acetate solution was reserved. The aqueous extracts, after back-extraction with ethyl acetate, were acidified; *α-benzyl* N-*benzoyl*-DL-*glutamate* separated (2·43 g., 33%), having m. p. 134—135°, raised to 136—137° by recrystallisation from 50% aqueous ethanol (Found: C, 67·2; H, 5·5; N, 4·1%; equiv., 336. $C_{19}H_{19}O_5N$ requires C, 66·8; H, 5·6; N, 4·1%; equiv., 341).

The ethereal mother-liquor above was extracted with an excess of N-ammonia (22 ml.), washed with water, combined with the ethyl acetate solution above, and evaporated to a gum (1·89 g.). This crystallised from ether–light petroleum, to give *dibenzyl* N-*benzoyl*-DL-*glutamate* (1·04 g., 11%), m. p. 80·5—82° (Found, in material dried at 65°: C, 72·4; H, 5·7; N, 3·5. $C_{26}H_{25}O_5N$ requires C, 72·4; H, 5·8; N, 3·3%).

Acidification of the ammoniacal solution above precipitated a semicrystalline mass (1·17 g.) which was dissolved in wet ethyl acetate (20 ml.) and extracted with 0·5N-sodium carbonate (3 ml., 2 × 2 ml., finally with 3 ml.). Precipitates were obtained from all four extracts by acidification. That from the first was extracted into ethyl acetate and recovered by evaporation as gum *B* (0·46 g.) which did not crystallise. The solids which separated from the second, third, and fourth extracts were collected separately (0·37 g., m. p. 124—127°; 0·238 g., m. p. 125—128°; 52 mg., m. p. 128—129°, the last unchanged in admixture with α-benzyl *N*-benzoyl-DL-glutamate).

1-*Benzyl*-5-(2-*benzyloxycarbonylethyl*)-2-*thiohydantoin* (XV).—Gum *B* from the foregoing experiment was warmed with acetic anhydride (2·3 ml.), glacial acetic acid (0·25 ml.), and ammonium thiocyanate (126 mg.) until a clear solution was obtained. This was kept at room temperature overnight, then evaporated to dryness, and the residue partitioned between ethyl acetate and dilute aqueous sodium hydrogen carbonate to afford a neutral fraction (162 mg.). Crystallised from aqueous alcohol, this gave 1-*benzoyl*-5-(2-*benzyloxycarbonylethyl*)-2-*thiohydantoin* as needles (78 mg., 32%), m. p. 124·5—125·5°, which were shown by sodium-fusion to contain sulphur (Found: C, 63·0; H, 4·7; N, 7·1. $C_{20}H_{18}O_4N_2S$ requires C, 62·8; H, 4·8; N, 7·3%).

When α-benzyl *N*-benzoyl-DL-glutamate (0·1 g.) was treated as above with acetic anhydride (0·5 ml.), glacial acetic acid (0·055 ml.), and ammonium thiocyanate (25·2 mg.), the total product gave, on crystallisation from 50% aqueous ethanol, only impure starting material (64 mg.), m. p. 118—121°, raised to 127—129° in admixture with starting material, m. p. 129—131°.

1-*Benzoyl*-5-*benzyloxycarbonyl*-DL-*pyrrolid*-2-*one* (IX*h*).—α-Benzyl *N*-benzoyl-DL-glutamate (2·3 g.) was treated with thionyl chloride (15 ml., 30 equiv.) at 0° as for the 5-ethoxycarbonyl analogue above, except that ethyl acetate was used for the isolation of the neutral product (2·06 g., 93%). This crystallised from ethyl acetate–light petroleum to give 1-*benzoyl*-5-*benzyloxycarbonyl*-DL-*pyrrolid*-2-*one* as needles (1·90 g.), m. p. 98—100° raised to 100—101° by repeated crystallisation from the same solvent mixture (Found, in material dried at 78°: C, 70·4; H, 5·4; N, 4·0. $C_{19}H_{17}O_4N$ requires C, 70·6; H, 5·3; N, 4·3%).

Dimethyl N-*Benzoylglycyl*-DL-*glutamate* (VIII*e*).—A stirred solution of *N*-benzoylglycine (14·32 g.) and triethylamine (11·2 ml.) in dioxan (500 ml.) and toluene (250 ml.) was treated at 0° with ethyl chloroformate (7·5 ml.). After 20 min., a solution of dimethyl DL-glutamate hydrochloride[11] (16·88 g.) and triethylamine (11·2 ml.) in dioxan (300 ml.) and water (30 ml.) was added and stirring was continued for 3 hr. while the cooling-bath warmed to room temperature. The organic solvents were evaporated and the resultant aqueous suspension was extracted with ethyl acetate (total 800 ml.). After being washed with 0·5N-hydrochloric acid (30 ml.), water, saturated aqueous sodium hydrogen carbonate (40 ml.), and finally water, the extract was dried and evaporated. The solid residue (17·02 g.) crystallised from ethyl

acetate–light petroleum, to give *dimethyl* N-*benzoylglycyl*-DL-*glutamate* as needles (15·71 g., 64%), m. p. 106—110° raised to 117—118° after sintering at 114° by repeated crystallisation from the same solvent mixture (Found: C, 57·0; H, 6·1; N, 8·0. $C_{16}H_{20}O_6N_2$ requires C, 57·1; H, 6·0; N, 8·3%).

N-*Benzoylglycyl*-DL-*glutamic Acid* (VIII*f*).—1·05N-Sodium hydroxide (267 ml.) was added to a solution of the foregoing diester (15 g.) in methanol (90 ml.), and the mixture was kept for 36 hr. at room temperature. After evaporation of the methanol, the solution was acidified. N-*Benzoylglycyl*-DL-*glutamic acid* separated as needles (12·51 g., 91%), m. p. 219—220°, unchanged by recrystallisation from water (Found: C, 54·4; H, 5·1; N, 9·4%; equiv., 159. $C_{14}H_{16}O_6N_2$ requires C, 54·5; H, 5·2; N, 9·1%; equiv., 154).

N-*Benzoylglycyl*-DL-*glutamic Acid* α- *and* γ-*Diethylamide* (I*d*) *and* (VII*d*).—Ethyl chloroformate (2 ml., 1 equiv.) was added to a stirred solution of the foregoing acid (6·16 g.) and triethylamine (2·8 ml., 1 equiv.) in chloroform (200 ml.) and dimethylformamide (110 ml.) at 0°. After 20 min., a solution of diethylamine hydrochloride (2·18 g., 1 equiv.) and triethylamine (2·8 ml.) in chloroform (90 ml.) was added and stirring was continued for 3 hr. at 0°. The solvents were then evaporated and the residue was partitioned between water (50 ml.) and ethyl acetate (200 ml., and, after filtration and separation, with 50 ml.). The undissolved solid (1·77 g.) crystallised from ethanol to give N-*benzoylglycyl*-DL-*glutamic acid* α-*diethylamide* as blades (1·54 g.), m. p. 182—184° raised to 183·5—184·5° by recrystallisation from the same solvent (Found: C, 59·4; H, 6·9; N, 11·7%; equiv., 356. $C_{18}H_{25}O_5N_3$ requires C, 59·5; H, 6·9; N, 11·6%; equiv., 363).

The combined ethyl acetate extracts above were shaken with an excess of aqueous potassium carbonate (5%, 30 ml.), and the alkaline extract was acidified to precipitate a solid (3·29 g.), m. p. 87—92° and equiv., 388. This was heated with ethyl acetate (250 ml.) and the undissolved solid *C* (0·94 g.), m. p. 114—130°, was reserved. The solution, after being saturated with water, was extracted with three portions of 0·5N-sodium hydroxide (3·1, 6·2, 5·2 ml.). These were acidified and the precipitates collected separately (298 mg., m. p. 85—88°; 970 mg., m. p. 90—91°; 322 mg., m. p. 84—86°, respectively). A solution of the 970 mg. crop in 0·5N-sodium hydroxide (5·65 ml., 1·05 equiv.) was treated with portions of N-hydrochloric acid (0·67, 2·2 ml.) to precipitate two fractions (0·17 g., m. p. 90—95°; 0·56 g., m. p. 87—91°). The latter was recrystallised four times from water to give N-*benzoylglycyl*-DL-*glutamic acid* γ-*diethylamide* as needles, m. p. 96—100° (Found, in material dried at 76°: C, 59·9; H, 6·9; N, 11·4%; equiv., 367).

The solid *C* above was shown to contain the isomeric α- and γ-diethylamides (I*d*) and (VII*d*) by two fractional precipitations with acid from a solution of the mixed sodium salts as in the preceding paragraph. The weaker acid (97 mg., m. p. 182—184°) and the stronger acid (56 mg., m. p. 92—95°) were identified by mixed m. p. determinations with pure samples.

1-*Benzoylglycyl*-5-*diethylcarbamoyl*-DL-*pyrrolid*-2-*one* (II*d*).—*N*-Benzoylglycyl-DL-glutamic acid α-diethylamide (I*d*) (1·28 g.) was treated with thionyl chloride (10 ml., 40 equiv.) at 0° for 17 hr. and worked up as for the 1-benzoyl-5-benzyloxycarbonyl analogue above. The neutral product (1·1 g., 90%) crystallised from ethyl acetate to give 1-*benzoylglycyl*-5-*diethylcarbamoyl*-DL-*pyrrolid*-2-*one* as prisms, m. p. 141—143° raised to 144·5—145·5° by recrystallisation from the same solvent (Found: C, 62·7; H, 6·6; N, 12·5. $C_{18}H_{23}O_4N_3$ requires C, 62·6; H, 6·7; N, 12·2%).

Alkaline Hydrolysis of 1-*Benzoylglycyl*-5-*diethylcarbamoyl*-DL-*pyrrolid*-2-*one* (II*d*).—A solution of the foregoing product (836 mg.) in dioxan (5 ml.) and water (2 ml.) was titrated with 0·1N-sodium hydroxide (26·8 ml., 1·1 equiv.) during 30 min. The solution was evaporated to dryness and the residue was extracted with hot ethyl acetate (60 ml.), followed by hot ether (30 ml.). The undissolved solid was dissolved in water and extracted with ethyl acetate (3 × 40 ml.), each extract being washed with water. All the aqueous solutions were combined (*ca.* 10 ml.) and treated with portions of N-hydrochloric acid (2 × 0·9 ml. and, after concentration of the solution to 5 ml., with 0·9 ml.), to yield three crops of crystals. The first (202 mg., 23%) had m. p. 179—181° raised to 182—183° in admixture with *N*-benzoylglycyl-DL-glutamic acid α-diethylamide (I*d*). The second crop was *N*-benzoylglycine (137 mg.), m. p. 182—183°, mixed m. p. 184—186°; in admixture with the peptide (I*d*) the m. p. was 156—160°. The third crop (60 mg.) was recrystallised twice from water, to give *N*-benzoylglycine (20 mg.), m. p. 181—182°, mixed m. p. 184—186° (total yield of *N*-benzoylglycine 157 mg., 36%).

Cyclisation of N-*Benzoyl*-α-DL-*glutamylglycine* n-*Hexylamide* (I*g*).—(a) *By thionyl chloride.* The glutamyl peptide (I*g*) (2 g.) was treated with thionyl chloride (7·3 ml., 20 equiv.) at 0° for 20 hr. and worked up as for 1-benzoyl-5-benzyloxycarbonyl-DL-pyrrolid-2-one above. The

mauve product (1·83 g., 96%), m. p. 152—154°, was crystallised from ethyl acetate, then from 35% aqueous ethanol (charcoal), and finally from ethyl acetate to give N-*benzoyl*-5-*oxo*-DL-*pyrrolidine*-2-*carbonylglycine* n-*hexylamide* (II*g*) as colourless needles, m. p. 160·5—161·5° (Found: C, 64·7; H, 7·2; N, 11·5. $C_{20}H_{27}O_4N_3$ requires C, 64·3; H, 7·3; N, 11·3%).

A portion (0·639 g.) of the neutral product, before purification, was fractionated by counter-current distribution (scattered in first 10 tubes), with the solvent system chloroform, benzene, methanol, 0·002N-aqueous hydrochloric acid in volume proportions 10 : 20 : 23 : 7. A trial sample of the solute had been recovered unchanged after being dissolved in this solvent system at room temperature for 30 hr. After 95 transfers, one major peak was obtained (*K* 0·8), and one minor one (*K* 0·07; *ca.* 30 mg.) which contained the coloured impurity. Tubes 0—33 were emptied and refilled with fresh solvent system, and the machine was then set for the recycling procedure.[18] Analyses were carried out after 508 and after 1060 transfers; only one peak, which fitted the theoretical curve, was found in each case. The solution from tubes 487—507 was freed from organic solvent, and the resultant aqueous suspension was extracted thrice with ethyl acetate. The combined extracts were shaken with an excess of 2% aqueous sodium hydrogen carbonate, dried, and evaporated. Crystallisation of the colourless residue from aqueous ethanol afforded the acylpyrrolidone (II*g*), m. p. 160—161°.

In separate experiments, the treatment of the α-glutamyl peptide (I*g*) with thionyl chloride as above was stopped after 2, 10, and 15 hr. and the yield of the acylpyrrolidone (II*g*) was 34, 94, and 94% respectively. After 19 hr. with thionyl chloride (20 equiv.) at −20° the peptide (I*g*) yielded 90% of the acylpyrrolidone (II*g*).

(b) *By the mixed carbonic anhydride method.* A stirred solution of *N*-benzoyl-α-DL-glutamylglycine *n*-hexylamide (2·63 g.) and triethylamine (1·35 ml.) in dioxan (210 ml.), toluene (105 ml.), and dimethylformamide (35 ml.) was treated at 0° with ethyl chloroformate (0·635 ml., 1 equiv.). After 20 min. at 0°, the solution was stirred for 2 hr. as the cooling-bath warmed to room temperature, and then was kept overnight. Water (100 ml.) was added gradually as the organic solvents were evaporated and the resultant suspension was shaken with ethyl acetate (total 450 ml.). Some insoluble acidic matter (406 mg.), m. p. *ca.* 170°, was removed, and the ethyl acetate solution was separated and extracted with an excess of aqueous sodium hydrogen carbonate (5%, 60 ml.). After being washed twice with water, the ethyl acetate solution was dried and evaporated, to give the neutral fraction (1·06 g.). DL-α-*Benzamidoglutaroylglycine* n-*hexylamide* was obtained as needles (491 mg., 37% based on unrecovered starting material), m. p. 206·5—207·5°, by crystallisation of the neutral fraction from ethanol (Found: C, 64·6; H, 7·3; N, 11·1. $C_{20}H_{27}O_4N_3$ requires C, 64·3; H, 7·3; N, 11·2%).

The aqueous alkaline solution above was acidified and the crystals were collected and dried *in vacuo* (1·23 g., 47%; m. p. 183—185° alone or in admixture with starting material).

Separation of the Imide (VI*g*) *from the Acylpyrrolidone* (II*g*).—A 1 : 1 mixture (62 mg.) of these two was crystallised from ethanol (1 ml.) to give the imide (VI*g*) (22 mg.), m. p. 206—207°, unchanged in admixture with the foregoing sample. Addition of water (0·5 ml.) to the mother-liquor gave a mixture of nodules (22 mg.), m. p. 150—154°, and needles (8 mg.), m. p. 193—198° after sintering at 160°, which were separated by hand. The former crop was recrystallised from aqueous ethanol, to give the acylpyrrolidone (II*g*), m. p. 152—156° raised to 154—158° in admixture with pure material.

1-*Benzoyl*-5-*oxo*-DL-*pyrrolidine*-2-*carboxylic Acid* (IX*i*).—A solution of 1-benzoyl-5-benzyloxycarbonyl-DL-pyrrolid-2-one (IX*h*) (1·66 g.) in a mixture of ethanol (150 ml.), glacial acetic acid (50 ml.), and water (5 ml.) was shaken with hydrogen and 10% palladised charcoal (1 g.) at 14°/764 mm. Uptake of hydrogen (1·0 mol.) was complete in 30 min. The filtered solution and washings were evaporated to dryness and the residue, in ethyl acetate (45 ml.), was shaken with aqueous sodium hydrogen carbonate (5%; 15 ml.). The alkaline extract was acidified immediately and the precipitated *acid* was collected (0·865 g.); it had m. p. 103—108°, then solidified and remelted at 133—134°. A further quantity (0·144 g., total yield 84%) with the same m. p. behaviour was obtained by extraction of the mother-liquors with ethyl acetate and crystallisation of the extract from water. For analysis, the first crop was recrystallised twice from water to give needles, m. p. 133—135°, after being dried at 78° *in vacuo* (Found: C, 61·3; H, 4·9; N, 6·0%; equiv., 234. $C_{12}H_{11}O_4N$ requires C, 61·7; H, 4·8; N, 6·0%; equiv., 233).

N-*Benzoyl*-5-*oxo*-DL-*pyrrolidine*-2-*carbonylglycine* n-*Hexylamide* (II*g*). The foregoing product (0·6 g.) and triethylamine (0·36 ml.) in dioxan (50 ml.) and toluene (15 ml.) were stirred at 0°

[18] Craig, Hausmann, Ahrens, and Harfenist, *Analyt. Chem.*, 1951, **23**, 1236.

with ethyl chloroformate (0·25 ml.) for 20 min. A solution of glycine *n*-hexylamide hydrochloride [3] (0·507 g.) and triethylamine (0·36 ml.) in dioxan (10 ml.) and water (0·75 ml.) was then added dropwise during 25 min. and stirring was continued for a further 1 hr. at 0°. After being kept overnight, the mixture was treated with water (50 ml.), and the organic solvents were evaporated. The resultant suspension was extracted with ethyl acetate (total 70 ml.), and the extract was washed with 0·01N-hydrochloric acid (2 × 15 ml.), water, aqueous sodium hydrogen carbonate (5%; 15 ml.), and finally with water. Evaporation of the dried extract left a solid (0·61 g.) which was recrystallised twice from aqueous ethanol, to give the acylpyrrolidone (II*g*) (0·36 g., 38%), m. p. 159—161°, not depressed in admixture with the sample from the thionyl chloride route above. The two samples also gave identical *X*-ray powder photographs. In admixture with the imide (VI*g*), the m. p. was depressed to 153—162°.

Alkaline Hydrolysis of N-*Benzoyl-5-oxo-*DL-*pyrrolidine-2-carbonylglycine* n-*Hexylamide* (II*g*).—The acylpyrrolidone (II*g*) [0·5 g., prepared by method (*a*) above] in dioxan (15 ml.) and water (10 ml.) was treated portionwise with 0·1N-sodium hydroxide (13·7 ml., 1·01 equiv.) during 20 min. After evaporation of the dioxan, the aqueous solution was extracted with ethyl acetate (2 × 100 ml.), and the extracts were washed with water, dried, and evaporated to leave a crystalline residue (166 mg., 45%). Recrystallisation twice from ethyl acetate–light petroleum gave impure 5-oxo-DL-pyrrolidine-2-carbonylglycine *n*-hexylamide as needles (116 mg.), m. p. 103—104° after sintering (Found, in material dried at 76°: C, 56·5; H, 9·1; N, 14·8. Calc. for $C_{13}H_{23}O_3N_3$: C, 57·9; H, 8·6; N, 15·6%).

The combined aqueous layers above were concentrated and acidified; a crystalline precipitate was collected, dried, and extracted with hot light petroleum (3 × 40 ml.), and the insoluble material *D* (0·18 g.), m. p. 140—151°, after sintering, was reserved. The aqueous solution was extracted with light petroleum (2 × 40 ml.), and the extract was dried, combined with the other petroleum solution, and evaporated, to leave benzoic acid (73 mg., 45%), m. p. and mixed m. p. 120·5—121°. Finally, the aqueous acid solution was extracted twice with ethyl acetate (total 180 ml.), and the extract, after being dried and evaporated, left a gum (51 mg.) which was combined with the solid *D* above. These acidic products were fractionated by countercurrent distribution (50 transfers) between ethyl acetate (4 vol.), butan-1-ol (1 vol.), and aqueous buffer (5·6 vol.) made from 0·5M-KH_2PO_4 (4 vol.) and 0·5M-K_2HPO_4 (2·8 vol.). Two separate peaks (*K* 0·47, 46%; *K* 2·7, 54%) were obtained. The solute from the former (tubes 8—25) was recovered as above and crystallised from 30% aqueous ethanol, to give *N*-benzoyl-γ-DL-glutamylglycine *n*-hexylamide [3] (VII*g*), m. p. and mixed m. p. 126—131°.

The solute from the second peak (tubes 29—45) was recovered and crystallised in the same way, to give *N*-benzoyl-α-DL-glutamylglycine *n*-hexylamide (I*g*), m. p. 182·5—183° raised to 183—183·5° in admixture with an authentic sample of m. p. 184—185°.

Hydrolysis in the same way of the total neutral fractions from the treatment (above) of the α-glutamyl peptide (I*g*) with thionyl chloride at 0° for 10 and 15 hr. and at −20° for 19 hr. yielded 42, 44, and 41% of benzoic acid, respectively.

When the acylpyrrolidone (II*g*) (0·25 g.), prepared as in the previous section, was hydrolysed and the products were worked up as above, benzoic acid (51%) and a neutral fraction (91 mg., 50%) were isolated. The total remaining acidic products (106 mg.) were shown to consist of the γ-glutamyl peptide (VII*g*) (45%) and the α-glutamyl peptide (I*g*) (55%).

Alkaline Hydrolysis of DL-α-*Benzamidoglutaroylglycine* n-*Hexylamide* (VI*g*).—A solution of the imide (VI*g*) (305 mg.) in dioxan (8 ml.) and water (1 ml.) was titrated with a 0·126N-sodium hydroxide (6·9 ml., 1·06 equiv.) during 25 min. and the products were worked up and fractionated as in the foregoing experiment. No benzoic acid was isolated and only a trace (11 mg.) of neutral material. The acidic products (297 mg., 93%) were shown as above to consist of a mixture of the γ-glutamyl peptide (VII*g*) (78%), m. p. 127—131° raised to 127·5—131·5° in admixture with a pure sample, and the α-glutamyl peptide (I*g*) (22%), m. p. and mixed m. p. 183—184°.

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