Studies on Specific Chemical Fission of Peptide Links. Part I. The Rearrangement of Aspartyl and Glutamyl Peptides.

By ALAN R. BATTERSBY and J. C. ROBINSON.

[Reprint Order No. 5736.]

The esters of α- and β-aspartyl peptides (types II and X; $n = 1$) have been converted by alkaline hydrolysis into mixtures of acidic aspartyl peptides of types (V) and (VI). In all cases studied, the former product preponderates. The rearrangement has been shown to occur by cyclisation to the related imides (IV) followed by hydrolysis. Esters of glutamyl peptides are rearranged in a similar way, but certain differences from the aspartyl analogues have been discovered. The possible application of this rearrangement in a scheme for the specific fission of a peptide chain at the aspartic and glutamic residues is discussed.

A PEPTIDE may be degraded for structural study by partial hydrolysis with acids or enzymes, or by stepwise removal of amino-acid residues from the ends of the chain. Advances in these fields were reviewed in 1952 by Khorana (*Quart. Reviews*, **6**, 340) and several more recent contributions have been made (*e.g.*, Boissonnas, *Helv. Chim. Acta*, 1952, **35**, 2226; Holley and Holley, *J. Amer. Chem. Soc.*, 1952, **74**, 5445; Kenner, Khorana, and Stedman, *J.*, 1953, 673). Since the structure of a small peptide is more readily elucidated by these methods than that of a large one, it is desirable that chemical methods should be devised for the fission of a peptide chain at known points along its length. A satisfactory method must give a high yield of fission products under mild conditions. The avoidance of racemisation at the α-carbon atoms of the amino-acid residues would be an advantage, but this is not of paramount importance for most degradative studies.

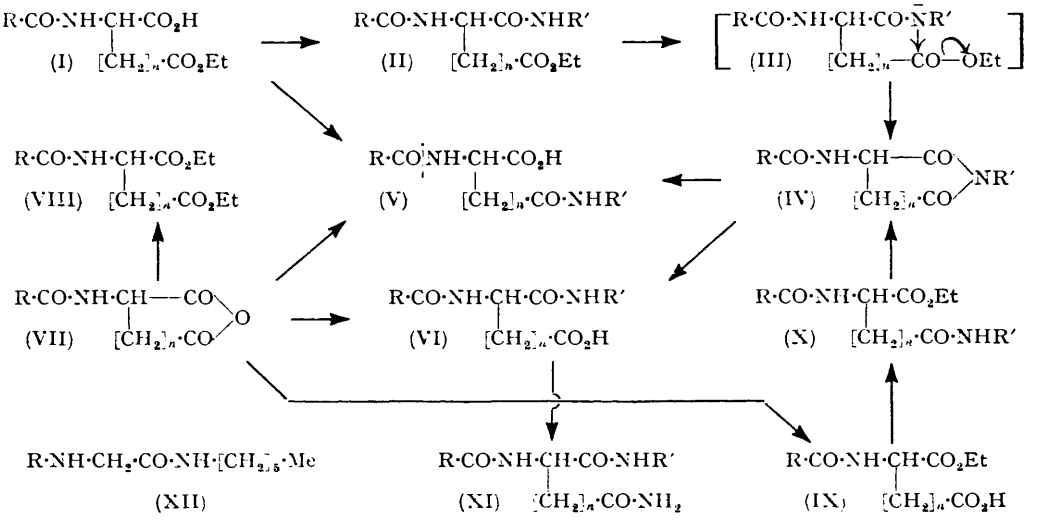
The only work along these lines seems to be that of Elliott (*Biochem. J.*, 1952, **50**, 542) who studied the specific hydrolysis of the peptide links of serine in silk fibroin. In our case, the initial effort has been directed towards a fission of the peptide chain at those points where aspartic or glutamic acid residues occur.

The first step of one scheme we envisaged, as briefly reported earlier (*Chem. and Ind.*, 1954, 45), involves the rearrangement of the normal α-aspartyl and α-glutamyl peptides (VI; $n = 1$ and 2) to give the β- and γ-isomers (V; $n = 1$ and 2) respectively. The latter possess the group $\cdots CO{\cdot}NH{\cdot}CHR{\cdot}CO_2H$ which differs from the carboxyl end of a peptide chain only in the nature of the group R. These peptides should therefore be susceptible to specific fission as indicated in (V) by use of one of the methods developed for selective removal of the *C*-terminal residue (Khorana; Boissonnas; Kenner, Khorana, and Stedman, *locc. cit.*). This second step will be the subject of a later communication.

Imides of type (IV) are the obvious intermediates for the required rearrangement, and their ready hydrolysis under mild alkaline conditions is well known (Titherley and Stubbs, *J.*, 1914, **105**, 299). It remained to establish that the desired hydrolysis product of type (V) was obtained in satisfactory yield. Cherbuliez and Chambers (*Helv. Chim. Acta*, 1925, **8**, 395) showed that hydrolysis of DL-benzamidosuccinimide (IV*a*) with hot saturated barium hydroxide affords *N*-benzoyl-DL-asparagine (V*a*) but the yield was not recorded. These conditions are unnecessarily drastic and we therefore hydrolysed (IV*a*) with cold dilute sodium hydroxide or equally effectively with warm 0·28N-sodium carbonate. Almost a quantitative yield was obtained of a product which was mainly the desired benzoylasparagine (V*a*), though a small amount of *N*-benzoyl-DL-*iso*asparagine (VI*a*) was also isolated. The latter was prepared for comparison by the action of ammonia on *N*-benzoyl-DL-aspartic anhydride (VII*a*) (cf. Pauly and Weir, *Ber.*, 1910, **43**, 661). Again a mixture of the two amides (V*a*) and (VI*a*) resulted, the latter preponderating. The relative amounts of (V*a*) and (VI*a*) obtained by ring-opening of the imide (IV*a*) and the anhydride (VII*a*) with hydroxyl ion and ammonia, respectively, must be governed, at least in part, by the relative electrophilic character of the two carbonyl groups in the ring. In the absence of

overpowering steric hindrance, attack at the α-carbonyl group (corresponding to the stronger carboxyl group) should be favoured, in accord with the results above and others given below (cf. Emery and Gold, *J.*, 1950, 1455).

Cherbuliez and Chambers (*loc. cit.*) prepared the imide (IV*a*) by heating benzoylasparagine at 200° and, as a by-product, they isolated an impure substance, m. p. 245—250°, which gave roughly the analysis for *N*-benzoyl-DL-aspartic diamide (XI*a*). We have now purified this material, m. p. 270—271°, and have confirmed the structure (XI*a*) by direct comparison with a specimen made from the imide (IV*a*) and ammonia. The drastic



Series (*a*) : n = 1, R = Ph, R′ = H.
Series (*b*) : n = 1, R = Ph, R′ = CH_2·CO·NH·[CH_2]$_5$·Me.
Series (*c*) : n = 2, R = Ph, R′ = H.
Series (*d*) : n = 2, R = Ph, R′ = CH_2·CO·NH·[CH_2]$_5$·Me.
Series (*e*) : n = 2, R = CH_2Ph·O·CO·NH·CH_2·CO·NH·CH_2, R′ = CH_2·CO·NH·[CH_2]$_5$·Me.
Series (*f*) : n = 2, R = NH$_2$·CH_2·CO·NH·CH_2, R′ = CH_2·CO·NH·[CH_2]$_5$·Me.

and evidently complex nature of the above preparation of imides makes it valueless for our present purpose. Attempts were therefore made to cyclise *N*-benzoyl-DL-*iso*asparagine ethyl ester (II*a*) under acidic conditions (see p. 264) but without success. A suitably mild method was discovered, however, when the action of 0·5 equiv. of dilute sodium hydroxide on the ester (II*a*) gave 43% of the imide (IV*a*); the latter was also formed when (II*a*) reacted with sodium ethoxide in ethanol. These reactions are probably initiated by removal of a proton to give (III*a*), followed by cyclisation. When an excess of cold 0·1N-sodium hydroxide or warm 0·28N-sodium carbonate was used, ring-closure of (II*a*) and subsequent hydrolysis of the imide were accomplished in one stage. A mixture of the asparagine (V*a*) and *iso*asparagine (VI*a*) derivatives was obtained, which was shown by fractionation on IR-4B resin to contain some 65% of the former.

In order to be sure that all the benzoylasparagine (V*a*) had been produced by rearrangement during the alkaline hydrolysis of (II*a*), it was necessary to prove that the supposed β-ethyl *N*-benzoyl-DL-aspartate (I*a*) used for the preparation of (II*a*) was in fact free from the α-ethyl isomer (IX*a*). The possibility of rearrangement in the conversion of the ester (I*a*) into (II*a*) by the mixed anhydride method (Boissonnas, *Helv. Chim. Acta*, 1951, **34**, 874; Vaughan, *J. Amer. Chem. Soc.*, 1951, **73**, 3547; Wieland and Bernhard, *Annalen*, 1951, **572**, 190) had also to be eliminated. First, the ester (I*a*) gave a good yield of benzoylasparagine (V*a*) when treated with aqueous ammonia. For further evidence, α-ethyl *N*-benzoyl-DL-aspartate (IX*a*) was prepared, in admixture with the β-isomer (I*a*) and the diethyl ester (VIII*a*), by heating *N*-benzoyl-DL-aspartic anhydride with ethanol. The α-ester was isolated almost pure by fractional extraction (Le Quesne and Young, *J.*, 1950, 1954) and was completely purified by countercurrent distribution (Craig and Craig,

" Technique of Organic Chemistry," Ed. by Weissberger, Interscience Publ., New York, 1950, Vol. III, p. 171). That our preparation of the β-ester (I*a*) was quite free from the α-ester (IX*a*) was shown by countercurrent distribution of the former alone and in admixture with the latter. A single peak was obtained in the first case and two in the second. Both pure isomers (I*a*) and (IX*a*) were converted by the mixed anhydride method into the corresponding amido-esters which were different and homogeneous and must be (II*a*) and (X*a*) respectively. Thus, rearrangement has not occurred by way of the mixed anhydrides but only in the treatment of the ester (II*a*) with bases. In this simple case, the desired conversion of an α-aspartyl " peptide " into the β-isomer has been achieved in good yield under mild conditions.

The ethyl esters (II*b*) and (X*b*) of *N*-benzoyl-α- and -β-DL-aspartylglycine *n*-hexylamide were prepared from (I*a*) and (IX*a*) respectively, by the mixed anhydride method using ethyl chloroformate and glycine *n*-hexylamide (XII; R = H). A by-product from these preparations was shown to be ethoxycarbonylglycine *n*-hexylamide (XII; R = CO_2Et) which could arise either by attack at the second carbonyl group of the mixed anhydride or from any ethyl chloroformate present in the reaction mixture as a result of incomplete mixed anhydride formation. The two esters (II*b*) and (X*b*) with warm aqueous sodium carbonate solution both gave mixtures of the isomeric acidic peptides (V*b*) and (VI*b*), which were readily separated by countercurrent distribution. The component which made up about 75% of each mixture was assigned the structure (V*b*) because it had a lower partition ratio than its isomer in the buffered solvent system and must be the stronger acid. This assignment was confirmed by acidic hydrolysis (Sheehan, Chapman, and Roth, *J. Amer. Chem. Soc.*, 1952, **74**, 3822) of the ester (II*b*), of unequivocal structure, to give (VI*b*), which was identical with the weaker acid obtained from the alkaline hydrolysis. Thus both esters are rearranged by alkali and the fact that they yield mixtures of much the same composition suggests that simple hydrolysis of the ester group is not a serious side reaction in the aspartic acid series. When the reaction with sodium carbonate was stopped before completion, the neutral product from both esters was the imide (IV*b*); hydrolysis of the imide is therefore the rate-controlling reaction. Finally, the structures of the acidic peptides (V*b*) and (VI*b*) were confirmed by synthesis from *N*-benzoyl-DL-aspartic anhydride and glycine *n*-hexylamide; the weaker acid (VI*b*) predominated in the product.

The asparaginyl peptide (XI*b*), prepared from the related acid (VI*b*) by way of the mixed (carbonic) anhydride, was largely unaffected by cold 0·1N-sodium hydroxide or warm 0·25N-sodium carbonate. The main part of the small amount of acidic material which was obtained was the rearranged peptide (V*b*).

In the glutamic acid series, *N*-benzoyl-α-DL-glutamylglycine *n*-hexylamide ethyl ester (II*d*) was more resistant to warm aqueous sodium carbonate solution than the aspartyl analogue (II*b*): 64% of the former ester was unchanged even after 7 hours. This difference in reactivity may be a valuable one. The glutamyl peptide was hydrolysed almost completely, however, by 0·1N-sodium hydroxide at room temperature to give a mixture of two acidic peptides, separable by countercurrent distribution. The main product (57%) was assigned the structure (V*d*) and the second product the structure (VI*d*) by the argument used in the aspartic acid series; the synthesis of these acidic peptides from *N*-benzoyl-DL-glutamic anhydride (VII*c*) and glycine *n*-hexylamide followed our earlier work on the aspartyl analogues. It is interesting that the yield of rearranged product from (II*d*) is appreciably lower than that from the aspartyl analogue (II*b*). One or both of the following factors may be involved: (*a*) the desired hydrolysis of the intermediate imide (IV*d*) to give (V*d*) is only slightly favoured over the alternative direction of ring-opening; (*b*) simple hydrolysis of the ester group in (II*d*) competes significantly with the ring-closure reaction. Further work is necessary on this point. In any case, esterification of the weaker acid (VI*d*) and treatment again with alkali would afford a better overall yield of the desired acid (V*d*). Clayton and Kenner (*Chem. and Ind.*, 1953, 1205) have briefly reported the conversion of a α-glutamyl peptide into the γ-isomer by a different method.

In the hydrolysis of (II*d*) with sodium hydroxide, a trace of neutral material was isolated and shown to be the unchanged ester; ring-closure to the imide is therefore the rate-

controlling step in contrast to the cases in the aspartic acid series. This is in accord with Sircar's observation (*J.*, 1927, 1252) that glutarimide is hydrolysed more readily than succinimide. It is not surprising then that our attempts to isolate the imide (IV*d*) were unsuccessful.

Finally, a study was made of the alkaline hydrolysis of the ethyl ester of an optically active peptide, *N*-acetylglycylglycyl-α-L-glutamylglycine *n*-hexylamide (II*f*). The main acidic product, optically inactive within the limits of experimental error, was assigned the structure (V*f*), *i.e.*, the rearranged product, because of its acidic strength (pK_a 3·45). This was confirmed by anodic oxidation of the main product in anhydrous methanol (Boissonnas, *Helv. Chim. Acta*, 1952, **35**, 2226) followed by complete hydrolysis of the oxidation products. Chromatography of the hydrolysate showed that the glutamic acid had been almost completely destroyed without the formation of a new amino-acid. In this it resembled the behaviour of the acid (V*d*) and differed from the acid (VI*d*) which were similarly oxidised for comparison. In the latter case, the glutamic acid was not completely destroyed and a new substance, probably α-amino-γ-methoxybutyric acid, was present in the hydrolysate. A minor acidic product, isomeric with (V*f*) but different from it, was also isolated from the alkaline hydrolysis of (II*f*); this product is probably the weaker acid (VI*f*) but the small quantity available and its awkward solubility properties precluded satisfactory identification.

It seems probable that the rearrangement described above is a general one for those esters of unsymmetrical succinamic and glutaramic acids which are capable of forming imides. Indeed, independently of our work, Hancock and Linstead (*J.*, 1953, 3490) observed rearrangement during alkaline hydrolysis of the anilic esters of methylsuccinic acid. More recently, Sondheimer and Holley (*J. Amer. Chem. Soc.*, 1954, **76**, 2467) have published other examples similar to ours in the amino-acid series. Syntheses involving alkaline hydrolysis of substances of this general type must therefore be used with care. For example, it seems likely that the acidic product obtained by alkaline hydrolysis of the polyester of α-polyglutamic acid (Bruckner, K. Kovács, J. Kovács, and Kótai, *Experientia*, 1954, **10**, 166) will contain an appreciable amount of γ-linked material.

Experimental

Analyses are mainly by Mr. B. S. Noyes of 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 acidifications, unless otherwise stated.

β-*Ethyl* N-*Benzoyl*-DL-*aspartate* (I*a*).—A solution of β-ethyl DL-aspartate hydrochloride (9·9 g., 0·05 mole) (Piutti, *Gazzetta*, 1888, **18**, 480; cf. Coleman, *J.*, 1951, 2294) and anhydrous potassium carbonate (10·4 g., 3 equiv.) in water (40 ml.) was stirred vigorously at 0° whilst benzoyl chloride (7 g.) in dioxan (20 ml.) was added during 1·5 hr. After being stirred for a further 2 hr. at 0°, the solution was made alkaline with potassium carbonate (0·3 g.) and extracted with ether (3 × 100 ml.). The aqueous layer was acidified and extracted with ethyl acetate (3 × 100 ml.). The latter extract, after being washed with water, dried, and evaporated, yielded a pale yellow gum (13·1 g.). When crystallised from 10% aqueous ethanol (300 ml.), this product gave β-*ethyl* N-*benzoyl*-DL-*aspartate* (9·99 g., 75%), m. p. 104—105°. A sample recrystallised twice from the same solvent gave diamond-shaped plates, m. p. 105—107° (Found: C, 58·9; H, 5·6; N, 5·0. $C_{13}H_{15}O_5N$ requires C, 58·9; H, 5·7; N, 5·3%). A portion of the final product was fractionated by countercurrent distribution (30 transfers) between ethyl acetate and aqueous phosphate buffer made from 0·5M-KH_2PO_4 (20 vol.) and 0·5M-K_2HPO_4 (1 vol.). One slightly skewed peak (partition ratio K 0·7) was obtained, indicating the presence of a single component for which K increases with increasing concentration. The presence of two components in a mixture of the β-ethyl ester (I*a*) and the α-ethyl ester (IX*a*) (K 2·2) was clearly visible after 30 transfers in the same solvent system.

γ-*Ethyl* N-*Benzoyl*-DL-*glutamate* (I*c*).—γ-Ethyl DL-glutamate, m. p. 187—188° (decomp.) (King and Spensley, *J.*, 1950, 3159), was prepared in 75% yield by the procedure used by Miller and Waelsch (*Arch. Biochem. Biophys.*, 1952, **35**, 176) for the L-isomer. The DL-ester (3 g.) was benzoylated and the product was crystallised as above to give γ-*ethyl* N-*benzoyl*-

DL-*glutamate* (3·21 g., 67%) as colourless needles, m. p. 112—114°, raised to 114—115·5° by recrystallisation twice from 10% aqueous ethanol (Found: C, 59·8; H, 6·0; N, 4·7%; equiv., 278. $C_{14}H_{17}O_5N$ requires C, 60·2; H, 6·1; N, 5·0%; equiv., 279).

N-*Benzoyl*-DL-*isoasparagine Ethyl Ester* (II*a*).—A stirred solution of the acid (I*a*) (5·3 g.) and triethylamine (2·8 ml.) in dioxan (250 ml.) and chloroform (50 ml.) was treated at 0° with ethyl chloroformate (2 ml.). An excess of aqueous ammonia (4 ml.; *d* 0·880) was added after 20 min. and stirring was continued for 5 hr. while the reaction mixture warmed to room temperature. After addition of water (50 ml.), the solution was made just acid to litmus and was evaporated to 50 ml.; the addition of water and evaporation were repeated. The resultant suspension was adjusted to pH 8 with potassium carbonate, and the solid collected and resuspended in 0·01N-hydrochloric acid. The insoluble *ethyl ester* (II*a*) (4·44 g., 83%), m. p. 149—150°, was collected and washed with water. One recrystallisation from 10% aqueous ethanol gave plates, m. p. 151—153°, unchanged by recrystallisation (Found: C, 59·3; H, 6·3; N, 10·2. $C_{12}H_{16}O_4N_2$ requires C, 59·1; H, 6·1; N, 10·6%).

Action of Ammonia on N-*Benzoyl*-DL-*aspartic Anhydride*.—A stream of dry ammonia was passed into a solution of the anhydride (3·6 g.) (Lawson, *J.*, 1953, 1046) in warm dry dioxan (160 ml.) until no further precipitate formed. The gummy ammonium salts, freed from dioxan by decantation, were dissolved in water (50 ml.), and the solution was acidified. A mixture of *N*-benzoyl-DL-asparagine (V*a*) and *N*-benzoyl-DL-*iso*asparagine (VI*a*) separated (3·94 g.), having m. p. 199—200° after sintering (Found, for mixture: equiv., 238. Calc.: equiv., 236). The whole crop was dissolved in 0·2N-sodium carbonate (90 ml., 1·1 equiv.) and portions of 1·15N-hydrochloric acid (11·7, 2·25, 5 ml.) were added to precipitate three fractions (2·32 g., m. p. 205—206°; 0·524 g., m. p. 194—196° after sintering from 180°; 0·591 g., m. p. 188—190°, respectively). The last was almost pure benzoylasparagine and melted at 189—190° in admixture with an authentic sample. The first fraction was again dissolved in aqueous sodium carbonate (1 equiv.), and hydrochloric acid (0·5 equiv.) was added; N-*benzoyl*-DL-iso-*asparagine* separated as needles, m. p. 208—209°, unchanged by recrystallisation from 10% aqueous ethanol (Found, in material dried at 110°: C, 55·8; H, 5·2; N, 12·0%; equiv., 243. $C_{11}H_{12}O_4N_2$ requires C, 55·9; H, 5·1; N, 11·9%; equiv., 236). This amide was recovered unchanged (90%) after being warmed for 3 hr. at 50—60° with 0·28N-sodium carbonate (3 equiv.).

N-*Benzoyl*-DL-*asparagine* (V*a*).—(a) *From* β-*ethyl* N-*benzoyl*-DL-*aspartate*. A solution of the ester (I*a*) (0·61 g.) in aqueous ammonia (40 ml.; *d* 0·880) was warmed at 40° for 3 days and then evaporated to dryness. The residue was dissolved in water (15 ml.) and acidified, *N*-benzoyl-DL-asparagine separating (0·51 g.), m. p. 184—186° raised to 191—192° by one recrystallisation from water. Cocker (*J.*, 1940, 1489) records m. p. 190—191°. Mixtures of this product with up to 30% of *N*-benzoyl*iso*asparagine melted at *ca.* 190—192°, after sintering; higher proportions of the *iso*asparagine raised the m. p., *e.g.*, 40% of the *iso*asparagine (VI*a*) with 60% of the asparagine derivative (V*a*) melted at 192·5—193°.

(b) *Racemisation of* N-*benzoyl*-L-*asparagine*. A solution of the L-amide (8·8 g.) (Pauly and Weir, *loc. cit.*) in water (53 ml.) was adjusted to pH 8 with sodium carbonate and then stirred with acetic anhydride (53 ml.) for 36 hr. without external cooling. Evaporation of the solution left a gum which was dissolved in water (50 ml.) and acidified. *N*-Benzoyl-DL-asparagine separated (8·3 g.), had m. p. 184—185°, raised by one recrystallisation from water to 191—192° alone or in admixture with the foregoing product (cf. Barker, *J.*, 1953, 453). A solution (*c*, 10·8) of the product in water containing ammonia (2 equiv.) was optically inactive.

N-*Benzoyl*-DL-*aspartic Acid Diamide* (XI*a*).—A solution of DL-benzamidosuccinimide (0·148 g.) in aqueous ammonia (10 ml.; *d* 0·880) deposited the *diamide* (0·13 g., 88%), m. p. 270—271° (decomp.), during 20 hr. at room temperature. Recrystallised from water, the diamide formed colourless needles (Found: C, 56·3; H, 5·6; N, 17·6. $C_{11}H_{13}O_3N_3$ requires C, 56·2; H, 5·6; N, 17·8%).

DL-*Benzamidosuccinimide* (IV*a*).—(a) *From* N-*benzoyl*-L-*asparagine*. Cherbuliez and Chambers's method (*loc. cit.*) yielded the two fractions described by them. One, sparingly soluble in ethanol, had m. p. 225—245°, raised by recrystallisation thrice from water to 268—269° alone or in admixture with the foregoing product. The second fraction, moderately soluble in ethanol, consisted of very crude imide (IV*a*) which in our hands could only be partly purified by repeated crystallisation from ethanol (cf. *idem*, *loc. cit.*). This fraction was therefore powdered finely and dissolved *rapidly* at room temperature in a slight excess of 0·25N-sodium carbonate, and the filtered solution was immediately adjusted to pH 7·5. Almost pure imide crystallised as prisms (3·5 g., 38%), m. p. 223—225°, raised by one recrystallisation from ethanol

to 224—225°. Cherbuliez and Chambers (*loc. cit.*) record m. p. 225—226°. *N*-Benzoyl-DL-*iso*-asparagine was cyclised in the same way to give the imide (IV*a*).

(b) *By the action of sodium hydroxide on* N-*benzoyl*-DL-iso*asparagine ethyl ester*. This ester (0·528 g.) was treated in ethanol (15 ml.) at room temperature with N-sodium hydroxide (1 ml., *ca.* 0·5 equiv.) and, after 18 hr., with 1·15N-hydrochloric acid (1·1 ml.). The alcohol was evaporated, water (10 ml.) added, and the resultant suspension brought to pH 7·5 with sodium carbonate. The neutral fraction (0·239 g.), m. p. 219—220° (after sintering), was collected, washed with water, and recrystallised from ethanol to give the imide (IV*a*) (0·189 g., 43%), m. p. 224—225° alone or in admixture with the foregoing product (Found, in material dried at 110°: C, 60·4; H, 4·7; N, 12·4. Calc. for $C_{11}H_{10}O_3N_2$: C, 60·5; H, 4·6; N, 12·8%).

(c) *By the action of sodium ethoxide on* N-*benzoyl*-DL-iso*asparagine ethyl ester*. A solution of the amido-ester (0·132 g.) in absolute ethanol (15 ml.) was warmed at 50° with sodium ethoxide (0·1 equiv.) for 8 hr. and then set aside for 30 hr. at room temperature. 0·1N-Hydrochloric acid (1 ml.) was added, and the solution evaporated to dryness. The crystalline residue was separated by partition as usual between ethyl acetate and aqueous sodium hydrogen carbonate into an acidic and a neutral fraction. The latter (0·121 g.), which flowed partly at 140—145° and was completely molten at 200°, was dissolved in hot water (20 ml.), and the cooled solution was filtered. An excess of silver nitrate (0·1 g.) was added to the filtrate, followed by dropwise addition of aqueous sodium carbonate solution until the immediate precipitation of white solid ceased. The silver derivative was collected, washed with water, and treated with dilute nitric acid; a clear solution was obtained which rapidly deposited crystals of the imide (IV*a*) (21 mg., 19%), m. p. 224—225° alone or in admixture with the foregoing product.

Attempts to cyclise benzoyl-DL-*iso*asparagine to the imide (IV*a*) with acetic anhydride at 100° were unsuccessful. *N*-Benzoyl-DL-*iso*asparagine ethyl ester was recovered unchanged after being heated at 180—190° for 0·5 hr.

Diethyl N-*Benzoyl*-DL-*aspartate* (VIII*a*).—*N*-Benzoyl-DL-aspartic acid (1 g.) (Cocker, *loc. cit.*) was treated in absolute ethanol (20 ml.) with dry hydrogen chloride and then heated under reflux for 1 hr. The *diester* (1·04 g., 84%), isolated in the usual way, crystallised from ether–light petroleum as rosettes of needles, m. p. 75—77° unchanged by recrystallisation (Found, in sample dried at 65°: C, 61·6; H, 6·5; N, 4·9. $C_{15}H_{19}O_5N$ requires C, 61·4; H, 6·5; N, 4·8%).

α-*Ethyl Hydrogen* N-*Benzoyl*-DL-*aspartate* (IX*a*).—*N*-Benzoyl-DL-aspartic anhydride (2·1 g.) in absolute ethanol (50 ml.) was heated under reflux for 3 hr. The gum recovered by evaporation of the solution was dissolved in ether and shaken with 0·28N-sodium carbonate (3 × 9 ml., finally with 20 ml.). The ether was dried and evaporated to leave a gum (0·214 g.) which crystallised from ether–light petroleum to give the diethyl ester (VIII*a*), m. p. 75—77° alone or in admixture with the foregoing product.

The four alkaline extracts when acidified precipitated oils, of which all save that from the first extract gradually crystallised; the solids from the second, third, and fourth extracts were collected separately (0·43 g., m. p. 103—104°; 0·566 g., m. p. 104—105°; 0·283 g., m. p. 104—106°, respectively). The combined crystalline product was freed from the β-ethyl isomer (I*a*) by countercurrent distribution (58 transfers) between ethyl acetate and the phosphate buffer described on p. 263. The solutions from tubes 38—51 were combined, and the separated aqueous layer was acidified and thoroughly extracted with ethyl acetate. After being washed with water, the upper layer from these tubes and the extracts were dried and evaporated to a gum (1·0 g.). This crystallised from water to give the α-*ethyl hydrogen ester* as prisms (0·785 g.), m. p. 111—112·5° (Found: C, 58·8; H, 5·7; N, 5·3. $C_{13}H_{15}O_5N$ requires C, 58·9; H, 5·7; N, 5·3%); the mixed m. p. with β-isomer (I*a*) was 80—95°.

N-*Benzoyl*-DL-*asparagine Ethyl Ester* (X*a*).—This was prepared on a 1 mmol. scale as for its isomer (II*a*) save that dioxan (22 ml.) and chloroform (4 ml.) were used to obtain a clear solution. The neutral products were extracted into ethyl acetate (total 110 ml.), and the extract was washed with water, dried, and evaporated to leave a white solid (0·208 g., 80%). Recrystallised from water, it yielded N-*benzoyl*-DL-*asparagine ethyl ester* as rosettes of long needles, m. p. 152—154° (Found: C, 59·3; H, 6·2; N, 10·4. $C_{13}H_{16}O_4N_2$ requires C, 59·1; H, 6·1; N, 10·6%). In admixture with its isomer (II*a*) the m. p. was 132—149°.

Attempted Cyclisation of Benzoyl-DL-iso*asparagine Ethyl Ester* (II*a*) *under Acidic Conditions*.—(*a*) A solution of anhydrous (II*a*) (0·1 g.) in anhydrous nitromethane (10 ml.) was treated with a slow stream of dry hydrogen bromide at 0° for 1 min. After being kept at room temperature in the dark for 16 hr., the yellow solution had deposited crystals (71 mg.) shown to be ammonium and/or methylamine hydrobromide derived from the nitromethane (cf. Albertson and McKay,

J. Amer. Chem. Soc., 1953, **75**, 5323). The nitromethane solution was evaporated to dryness and the residue was partitioned between aqueous sodium hydrogen carbonate and ethyl acetate. The neutral fraction (84 mg.) was very soluble in ethanol (1 ml.) and did not crystallise on seeding with the imide (IV*a*).

(*b*) Dry hydrogen chloride was bubbled for 5 min. into a solution of the ester (II*a*) (0·1 g.) in absolute ethanol (35 ml.) at 0°. After being kept for 16 hr. at room temperature, the solution was evaporated to a gum which was separated into two fractions by treatment with ether. One, readily soluble (40 mg.), was the crude diethyl ester (VIII*a*), m. p. 70·5—72·5° raised to 72·5—74·5° in admixture with an authentic sample. The second, sparingly soluble (39 mg.), was a mixture of ammonium chloride and starting material.

Action of Bases on DL-*Benzamidosuccinimide* (IV*a*).—A solution of the imide (0·436 g.) in 20% aqueous ethanol (20 ml.) was treated with N-sodium hydroxide (2·1 ml., 1·05 equiv.) and kept for 16 hr. at room temperature. After extraction with ethyl acetate (3 × 50 ml.) to remove neutral matter (28 mg.), the solution was adjusted to pH 7·5 with hydrochloric acid and concentrated to 5 ml. This was acidified to precipitate the acidic fraction (0·441 g., 94%), shown to be largely *N*-benzoyl-DL-asparagine by the m. p. 190—192° (decomp.; after sintering). The whole fraction was dissolved in aqueous sodium hydroxide (3 ml., 1 equiv.) and precipitated fractionally with portions (0·2 ml.) of 1·16N-hydrochloric acid. The first two fractions were combined (73 mg.), m. p. 197—199°, redissolved in aqueous sodium carbonate (1·5 ml., 1 equiv.), and precipitated with hydrochloric acid (0·5 equiv.). Slightly impure *N*-benzoyl-DL-*iso*asparagine separated (24 mg.), m. p. 202—204° raised to 205—207° in admixture with a pure specimen.

The imide (0·1 g.) was also hydrolysed quantitatively by being warmed with 0·28N-sodium carbonate (5 ml., *ca.* 3 equiv.) at 50° for 3 hr. The product (0·11 g.), m. p. 190—191° (decomp.; after sintering), was similar to that above.

Action of Bases on N-*Benzoyl*-DL-iso*asparagine Ethyl Ester* (II*a*).—This ester (0·1 g.) was warmed with 0·28N-sodium carbonate (4·2 ml., 3 equiv.) at 40° to give a clear solution after 2 hr. After a further 2 hr., the cooled solution was adjusted to pH 7, extracted with ethyl acetate (3 × 15 ml.), concentrated to 4 ml., and acidified. The product crystallised as needles (85 mg.), m. p. 190—192°, after sintering at 175°. A portion (9·9 mg.) in water (10 ml.) was run on to a column (1 cm. × 15 cm.) of IR-4B resin which had been washed thoroughly with N-hydrochloric acid and distilled water. The column was eluted with N-acetic acid (50 ml.) and then with N-hydrochloric acid (30 ml.). The latter eluted the asparagine derivative (V*a*) (6·4 mg., 65%). Trial experiments with synthetic mixtures of the two amides (V*a*) and (VI*a*) had shown that complete separation was achieved under these conditions, the weaker acid (VI*a*) being eluted by the acetic acid.

A suspension of the ester (II*a*) in 0·11N-sodium hydroxide (1·1 equiv.) was shaken at room temperature for 1 hr. to give a clear solution. This yielded a similar product to that above on being worked up in the same way.

Glycine n-*Hexylamide* (XII; R = H).—*n*-Hexylamine (6·28 g., 2 mol.) in dioxan (30 ml.) was added during 40 min. to a stirred solution of phthaloylglycyl chloride (6 g.) (Sheehan and Frank, *J. Amer. Chem. Soc.*, 1949, **71**, 1856) in dioxan (100 ml.) and chloroform (20 ml.) at 0°. The solution was evaporated to one-third bulk, and aqueous sodium hydrogen carbonate (0·5%, 200 ml.) was added. The precipitated solid, m. p. 163—165°, was washed with water and recrystallised from 50% aqueous ethanol (200 ml.) to give *phthaloylglycine* n-*hexylamide* as long needles (5·7 g., 64%), m. p. 164—166° raised by further recrystallisation from the same solvent to 166—167° (Found: C, 66·5; H, 7·2; N, 10·2. $C_{16}H_{20}O_3N_2$ requires C, 66·6; H, 7·0; N, 9·7%).

A solution of the foregoing product (32·4 g.) in ethanol (700 ml.) was heated under reflux with N-ethanolic hydrazine hydrate (113 ml.) for 1 hr. The cooled solution was filtered and evaporated to dryness. Treatment of the residue with warm N-hydrochloric acid (600 ml.) and removal of the precipitated phthalhydrazide gave a clear solution which was evaporated to a gum. After being thoroughly dried (P_2O_5), the gum crystallised from ethanol–ethyl acetate (1 : 10) to give *glycine* n-*hexylamide hydrochloride* as hygroscopic plates (11·35 g., 52%), m. p. 249° (decomp.; after considerable sintering at 95°) (Found, in material dried at 78°: C, 49·3; H, 10·0%; equiv., 199. $C_8H_{19}ON_2Cl$ requires C, 49·3; H, 9·8%; equiv., 194·5).

γ-Ethyl Benzyloxycarbonylglycylglycyl-L-*glutamate* (I*e*).—After many experiments in which the effect of temperature, reaction time, and solvent was studied, the following satisfactory procedure was used. A vigorously stirred solution of benzyloxycarbonylglycylglycine (21·7 g.) (Bergmann and Zervas, *Ber.*, 1932, **65**, 1192) and triethylamine (12 ml.) in chloroform (210 ml.) was treated at 0° with ethyl chloroformate (7·8 ml.). After 25 min., a solution of γ-ethyl

L-glutamate (21·4 g.) (Miller and Waelsch, *loc. cit.*) in 0·1N-sodium hydroxide (118 ml.) was added and stirring was continued for 2 hr. at 0° and 2 hr. at room temperature. After being filtered, the aqueous and the chloroform layer were separated and the latter was washed with aqueous ammonia (1·5%, 2 × 100 ml.) and water (100 ml.). Evaporation of the dried chloroform layer left a neutral gum which was not further examined. All the aqueous solutions were combined and acidified. The precipitated oil rapidly crystallised, and the solid was recrystallised from water (170 ml.) to give the *tripeptide* (I*e*) (8·4 g., 24%), m. p. 144—146°, unchanged by recrystallisation from water (Found: C, 53·9; H, 6·1; N, 10·0%; equiv., 424. $C_{19}H_{25}O_8N_3$ requires C, 53·9; H, 6·0; N, 9·9%; equiv., 423).

The tripeptide (I*e*) (2 g.) was fractionated by counter current distribution (100 transfers) between *sec.*-butanol and aqueous phosphate buffer made from 0·5M-KH_2PO_4 (9 vol.) and 0·5M-K_2HPO_4 (1 vol.). A single peak (*K* 2·45) was obtained which fitted the theoretical curve (Craig and Craig, *loc. cit.*). The tripeptide, recovered as in previous cases from tubes 64—83, was obtained as needles (1·5 g.), m. p. 151—153°, by crystallisation from water. When chromatographed on Whatman No. 1 paper with *sec.*-butanol (100 vol.) and aqueous ammonia (3%, 40 vol.) as the solvent system, this product had R_F 0·61; benzyloxycarbonylglycylglycine had R_F 0·38 in the same solvent.

Ethoxycarbonylglycine n-*Hexylamide* (XII; R = CO_2Et).—Ethyl chloroformate (0·1 ml.) was added to a solution of glycine *n*-hexylamide hydrochloride (0·2 g.) and triethylamine (0·35 ml.) in warm dioxan (10 ml.). After 0·5 hr. at 50°, the solution was poured into ethyl acetate (100 ml.) and extracted successively with portions (10 ml.) of N-hydrochloric acid, water, saturated aqueous sodium hydrogen carbonate, and finally water. The dried ethyl acetate solution was evaporated, and the residue crystallised from aqueous ethanol as large plates, m. p. 94—95°. A sample of the *amide* was sublimed at 85° (bath)/0·01 mm. for analysis (Found: C, 57·1; H, 9·7; N, 12·5. $C_{11}H_{22}O_3N_2$ requires C, 57·3; H, 9·6; N, 12·2%).

General Method for Preparation of Glycine n-*Hexylamides of Acyl Aspartic and Glutamic Acid Mono-esters.*—A solution of the mono-ester (5 mmol.) in dioxan (80 ml.) and chloroform (15 ml.) was stirred at 0° with triethylamine (0·7 ml.) and ethyl chloroformate (0·48 ml.). After 20 min., a further portion (0·7 ml.) of triethylamine was added, followed immediately by a solution of glycine *n*-hexylamide hydrochloride (5 mmol.) in dioxan (15 ml.) and water (1 ml.). The mixture was stirred for 2 hr. at 0°, then 2 hr. at room temperature, and finally adjusted to pH 6 with hydrochloric acid. Water (50 ml.) was added and the chloroform and dioxan were evaporated. The oil which separated was extracted into ethyl acetate (3 × 100 ml.) and the extract was washed with 2N-hydrochloric acid (10 ml.), saturated aqueous sodium hydrogen carbonate (15 ml.), and finally with water. Evaporation of the dried extract left the coupled product as a gum. Unchanged monoester (*ca.* 25%) was recovered from the alkaline extracts by acidification. The following peptides were prepared by this method:

(a) N-*Benzoyl*-α-DL-*aspartylglycine* n-*hexylamide ethyl ester* (II*b*) crystallised as long needles (80% based on unrecovered monoester), m. p. 99—102° raised by recrystallisation thrice from aqueous ethanol to 105—107° (Found: C, 62·5; H, 7·6; N, 10·4. $C_{21}H_{31}O_5N_3$ requires C, 62·2; H, 7·7; N, 10·4%). The mother-liquor from the first crystallisation of the peptide (II*b*) was concentrated to low bulk, and a solid separated (0·14 g.). This was recrystallised thrice from aqueous ethanol to give large plates, m. p. 93—95° raised to 94—95° in admixture with authentic ethoxycarbonylglycine *n*-hexylamide above.

(b) N-*Benzoyl*-β-DL-*aspartylglycine* n-*hexylamide ethyl ester* (X*b*) (60%) crystallised as fine needles, m. p. 82—87° after sintering (Found, in material dried at 56°: C, 61·9; H, 7·7; N, 10·1. $C_{21}H_{31}O_5N_3$ requires C, 62·2; H, 7·7; N, 10·4%).

(c) N-*Benzoyl*-α-DL-*glutamylglycine* n-*hexylamide ethyl ester* (II*d*) crystallised as felted needles (84%), m. p. 108—109° raised by three recrystallisations from aqueous ethanol to 110—112° (Found: C, 63·1; H, 7·9; N, 10·2. $C_{22}H_{33}O_5N_3$ requires C, 63·0; H, 7·9; N, 10·0%).

(d) N-*Benzyloxycarbonylglycylglycyl*-α-L-*glutamylglycine* n-*hexylamide ethyl ester* (II*e*) was prepared as above save that the mono ester (I*e*) (5 mmole) was dissolved in dioxan (50 ml.) and toluene (35 ml.) to avoid racemisation (Vaughan, *J. Amer. Chem. Soc.*, 1952, **74**, 6137). The peptide crystallised from aqueous ethanol as rosettes of needles (76%), m. p. 142—147°, after sintering, $[\alpha]_D^{18}$ +3·6° (*c*, 4·88 in EtOH) (Found: C, 57·6; H, 7·3; N, 12·2. $C_{27}H_{41}O_8N_5$ requires C, 57·5; H, 7·3; N, 12·4%).

N-*Benzoyl*-α- *and* -β-DL-*aspartylglycine* n-*Hexylamides* (VI*b*) *and* (V*b*).—A solution of glycine *n*-hexylamide hydrochloride (0·72 g.) and triethylamine (1·05 ml., 2 equiv.) in dioxan (35 ml.) and chloroform (10 ml.) was warmed at 40° for 4 hr. with *N*-benzoyl-DL-aspartic anhydride (0·82 g.). The solvents were evaporated with occasional addition of water (total 40 ml.), and

the final aqueous solution was acidified and extracted with ethyl acetate (3 × 70 ml.). After being washed with water, the extract was shaken with an excess of 2N-aqueous ammonia. The alkaline solution was acidified, and the oil which was precipitated gradually crystallised (1·09 g., 77%), m. p. 150—162°. A sample (0·3 g.) was fractionated by countercurrent distribution (40 transfers) between ethyl acetate (8 vol.), *n*-butanol (2 vol.), and aqueous phosphate buffer (11·2 vol.) made from 0·5M-KH_2PO_4 (11 vol.) and 0·5M-K_2HPO_4 (1·6 vol.). Two completely separated peaks were obtained (*K* 1·05, 43%; *K* 5·15, 57%). The aqueous layer from tubes 13—27 (*K* 1·05) was acidified and then shaken with the organic layer from the same tubes, followed by two extractions with ethyl acetate. The combined organic extracts were shaken with an excess of aqueous sodium carbonate solution and the separated aqueous phase was acidified. N-*Benzoyl*-β-DL-*aspartylglycine* n-*hexylamide* (V*b*) separated as needles (88 mg.), m. p. 163—164° (decomp.), unchanged by recrystallisation from water (Found: C, 60·5; H, 7·2; N, 10·9%; equiv., 372. $C_{19}H_{27}O_5N_3$ requires C, 60·5; H, 7·2; N, 11·1%; equiv., 377). N-*Benzoyl*-α-DL-*aspartylglycine* n-*hexylamide* (VI*b*), recovered in the same way from tubes 30—42 (*K* 5·15), formed needles (111 mg.), m. p. 179—180° (decomp.), unchanged by recrystallisation from water (Found: C, 60·3; H, 7·1; N, 10·9%; equiv., 368).

The ester (II*b*) (1 g.) was boiled with acetone (12·5 ml.), water (8·5 ml.), and concentrated hydrochloric acid (3·8 ml.) for 2 hr. and the products were separated into an acidic and a neutral fraction as usual. The former, crystallised from water, gave the weaker acid (VI*b*) (0·384 g., 40%), m. p. 176—176·5° raised to 176·5—177° in admixture with the foregoing sample.

N-*Benzoyl*-DL-*asparaginylglycine* n-*Hexylamide* (XI*b*).—This was prepared from the related acid (VI*b*) on the 1 mmole scale as for the amide (II*a*) save that dioxan (22 ml.) and chloroform (4 ml.) had to be used to obtain a clear solution. The *asparaginyl peptide* (XI*b*) crystallised from aqueous ethanol as fine needles (66%), m. p. 184—185·5° raised to 186—187° by one recrystallisation from the same solvent (Found: C, 60·1; H, 7·6; N, 15·3. $C_{19}H_{28}O_6N_4$ requires C, 60·6; H, 7·5; N, 14·9%).

The ester (II*b*) when treated with saturated alcoholic ammonia solution at room temperature for 4 days gave a neutral fraction from which the asparaginyl peptide (XI*b*) was isolated (12%) by crystallisation from aqueous ethanol. The m. p. 183—184° was unchanged in admixture with the foregoing product.

N-*Acetylglycylglycyl*-α-L-*glutamylglycine* n-*Hexylamide Ethyl Ester* (II*f*).—A solution of the benzyloxycarbonyl tetrapeptide (II*e*) (6 g.) in methanol (250 ml.) containing glacial acetic acid (0·8 ml.) was shaken with hydrogen and palladium oxide (0·4 g.) until the evolution of carbon dioxide ceased. The filtered solution was evaporated to a gum which was treated with water (200 ml.), and the insoluble matter (1·71 g.) was filtered off. The latter crystallised from aqueous ethanol to afford unchanged starting material (1·55 g.), m. p. 138—142° (sintering at 120°), raised to 140—144° (after sintering) in admixture with authentic (II*e*). The aqueous filtrate above was shaken with acetic anhydride (160 ml.) for 20 min. with cooling to keep the temperature below 45°. A second portion (30 ml.) of acetic anhydride was added and, after being shaken for 5 min., the solution was set aside for 3·5 hr. Evaporation left a gum which crystallised from water (70 ml.) to give the *acetyltetrapeptide* (II*f*) (1·2 g.), m. p. 190—194°, after sintering. Treatment of the mother-liquor from this crop with acetic anhydride (60 ml.) as before gave a second crop (0·6 g.) of (II*f*), m. p. 188—195°, and concentration of the final mother-liquor gave a third crop (0·64 g.), m. p. 187—189° (total yield 2·44 g., 65% based on unrecovered starting material). The first crop, recrystallised twice from water gave needles, m. p. 193·5—194·5° (Found: C, 53·9; H, 8·0; N, 15·1. $C_{21}H_{37}O_7N_5$ requires C, 53·5; H, 7·9; N, 14·9%).

Action of Bases on the Esters and Amides of Aspartyl Peptides.—(a) *On* N-*benzoyl*-α-DL-*aspartylglycine* n-*hexylamide ethyl ester* (II*b*). This ester (0·71 g.) was stirred with 0·28N-sodium carbonate (25 ml., 4 equiv.) at 40°. After 2 hr., the undissolved oil was removed, dissolved in ethanol (0·5 ml.), and returned to the reaction mixture. A cloudy solution was obtained which rapidly became clear. After 4 hr., the cooled solution was adjusted to pH 7, freed from neutral matter (12 mg.) by extraction with ethyl acetate, and acidified. The precipitated oil rapidly crystallised (0·63 g., 95%); it had m. p. 155—157° (decomp.; after sintering). A portion (0·3 g.) of this product was fractionated by countercurrent distribution (40 transfers) in the same solvent system as used above for the separation of the acidic aspartyl peptides (V*b*) and (VI*b*). The same two peaks were obtained. From one (*K* 1·05, 75%), the stronger acid (V*b*) was isolated as before, having m. p. 160—162° (decomp.) alone or in admixture with the earlier sample. The other peak (*K* 5·6, 25%) yielded the weaker acid (VI*b*), m. p. 177—179° (decomp.) raised to 178—180° in admixture with the earlier sample.

In a second experiment on the same scale, the material (0·15 g.) undissolved after 2 hr. was collected and washed with water. It crystallised from aqueous ethanol to afford DL-*benzamidosuccinoylglycine* n-*hexylamide* (IV*b*) as stout prisms, m. p. 149—150° (Found: C, 63·1; H, 7·1. $C_{19}H_{35}O_4N_3$ requires C, 63·5; H, 7·0%).

(b) *On* N-*benzoyl*-β-DL-*aspartylglycine* n-*hexylamide ethyl ester* (X*b*). This was treated as above on $\frac{1}{5}$-scale and the material (37 mg.) undissolved after 3·5 hr. was crystallised from aqueous ethanol to give the imide (IV*b*), m. p. 148—149° alone or in admixture with the foregoing product. The mother-liquors from the imide were added to the main alkaline solution, which was then warmed at 50° for a further hour. The acidic products (91 mg.) were isolated as before and fractionated by countercurrent distribution (40 transfers) in the solvent system used in the previous experiment. Two peaks (*K* 1·05, 77%; *K* 5·15, 23%) were obtained corresponding to the isomeric acids (V*b*) and (VI*b*) respectively. It was confirmed that the latter band contained the weaker acid (VI*b*) by isolation of the solute from tubes 29—38 as earlier; m. p. 175·5—176° raised to 176—177° in admixture with an authentic sample.

(c) *On* N-*benzoyl*-DL-*asparginylglycine* n-*hexylamide* (XI*b*). Ammonia was slowly evolved when this peptide (0·1 g.) was shaken vigorously with 0·1N-sodium hydroxide (8 ml., 3 equiv.) at room temperature for 6 hr. Unchanged starting material (82 mg.) was filtered off, and the acidic products (14 mg.), m. p. 143—144·5°, were recovered from the filtrate as before.

A second portion of the peptide (XI*b*) (0·1 g.) was heated for 6 hr. at 50° with 0·25N-sodium carbonate (3·2 ml., 3 equiv.). The products were worked up as before to afford recovered starting material (79 mg.) and an acidic fraction (14 mg.), m. p. 149—151°.

The two portions of acidic material were combined and fractionated by countercurrent distribution (40 transfers) in the solvent system used in the previous experiment. The areas under the two peaks obtained (*K* 1·0 and 5·1) showed that the two components were present approximately in the ratio 3 : 1 respectively. The solute recovered from tubes 15—26 (*K* 1·0) was the stronger acid (V*b*), m. p. 157·5—158° alone or in admixture with an authentic sample.

Action of Bases on the Esters of Glutamyl Peptides.—(a) *On* N-*benzoyl*-α-DL-*glutamylglycine* n-*hexylamide ethyl ester* (II*d*). The peptide (0·419 g.) was stirred with 0·25N-sodium carbonate (16 ml., 4 equiv.) at 50° for 7 hr. Unchanged material (0·269 g.) was filtered off, and the acidic products (0·136 g.), m. p. 123—150°, were recovered from the filtrate as usual.

In a second experiment, the peptide (II*d*) (0·419 g.) was shaken vigorously at room temperature with 0·1N-sodium hydroxide (18 ml., 1·8 equiv.) and after 4 hr. a trace of starting material (5 mg.), m. p. 108—109°, was filtered off. The acidic products (0·361 g., 93%), m. p. 125—155°, were isolated as usual and fractionated by countercurrent distribution (40 transfers) between ethyl acetate (4 vol.), *n*-butanol (1 vol.), and aqueous phosphate 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* 0·45, 57%; *K* 2·8, 43%) were obtained. The solute from the former (tubes 7—19) was recovered as usual and crystallised from 30% aqueous ethanol to give N-*benzoyl*-γ-DL-*glutamylglycine* n-*hexylamide* (V*d*) as needles (135 mg.), m. p. 129—131° (after sintering), unchanged by recrystallisation from the same solvent (Found: C, 61·1; H, 7·4; N, 10·8. $C_{20}H_{29}O_5N_3$ requires C, 61·4; H, 7·5; N, 10·7%).

The solute from the second band (tubes 24—36) was recovered and crystallised in the same way to give N-*benzoyl*-α-DL-*glutamylglycine* n-*hexylamide* (VI*d*) as shining plates (0·107 g.), m. p. 183—185°, raised to 184—186° by one recrystallisation from 30% aqueous ethanol (Found: C, 61·6; H, 7·4; N, 10·3%).

The ester (II*d*) was treated at room temperature in ethanol with N-sodium hydroxide (0·3 equiv.) for 4 days, and in a second experiment with sodium ethoxide (1 equiv.) for 1 week, but in neither case could the imide (IV*d*) be isolated from the neutral products.

(b) *On* N-*acetylglycylglycyl*-α-L-*glutamylglycine* n-*hexylamide ethyl ester* (II*f*). This ester (2·31 g.) was shaken with 0·1N-sodium hydroxide (62 ml., 1·2 equiv.), and after 6 hr. at room temperature, the clear solution was extracted with ethyl acetate (2 × 50 ml.). The aqueous layer was adjusted to pH 5, concentrated to 15 ml., and acidified. N-*Acetylglycylglycyl*-γ-DL-*glutamylglycine* n-*hexylamide* (V*f*) separated as plates (1·22 g., 56%), m. p. 186—190° raised to 194·5—195·5° by recrystallisation from water, $[\alpha]_D^{18}$ 0° ± 0·1° (*c*, 12·4 in 1·2 equiv. of aqueous NaOH) (Found: C, 51·5; H, 7·4; N, 15·6%; equiv., 439. $C_{19}H_{33}O_7N_5$ requires C, 51·5; H, 7·5; N, 15·8%; equiv., 443); it had pK_a, 3·45 by potentiometric titration in water.

The mother-liquor from (V*f*) was concentrated to yield a second crop of acidic matter 0·508 g.), m. p. 185—187° (after sintering), and the final mother-liquor was evaporated to a gum. The latter was worked through dimethylformamide, ethanol, and ethyl acetate to remove inorganic salt, and finally obtained as an amorphous solid (182 mg.) which contained an

appreciable amount of chloride ion but no sodium ion. The second crop above, recrystallised from water, gave needles (293 mg.) (Found : equiv., 446), m. p. 194—195·5° depressed in admixture with (V*f*). A portion of this material was completely hydrolysed by being heated with 6N-hydrochloric acid for 20 hr. at 110°. The presence in the hydrolysate of glutamic acid, glycine, and *n*-hexylamine was shown by paper chromatography.

N-*Benzoyl*-DL-*glutamic Anhydride* (VII*c*).—Anhydrous *N*-benzoyl-DL-glutamic acid (13 g.) (Fischer, *Ber.*, 1899, **32**, 2464) was heated at 100° with acetic anhydride (40 ml.) until a clear solution resulted and then for a further 10 min. The solution was cooled to 0°, and the *anhydride* (10·5 g., 87%) collected and washed with anhydrous light petroleum. Recrystallised from acetic anhydride, it formed large plates, m. p. 154—155° (Found : C, 62·2; H, 4·9; N, 6·0. $C_{12}H_{11}O_4N$ requires C, 61·8; H, 4·8; N, 6·0%).

N-*Benzoyl- and* -α-γ-DL-*glutamylglycine* n-*Hexylamides* (VI*d*) *and* (V*d*).—These were prepared from glycine *n*-hexylamide hydrochloride (0·98 g.) and benzoylglutamic anhydride (1·17 g.) in the way used above for the aspartyl analogues. During the isolation of the acidic products as before, a portion (0·45 g.) failed to dissolve in ethyl acetate and was shown to be the pure weak acid (VI*d*) by its m. p. 184—186° alone or in admixture with the sample above. The remaining acidic material in the ethyl acetate was shaken with 0·25N-sodium carbonate (3 × 10 ml.) and finally with an excess of 2N-aqueous ammonia. The second and the third carbonate extract were combined and acidified. The precipitated solid (42 mg.) was recrystallised from 30% aqueous ethanol to afford the stronger acid (V*d*), m. p. 127—129° raised to 128—130° in admixture with the pure sample obtained as above. The ammoniacal solution, when acidified, precipitated a mixture of the two acids (V*d*) and (VI*d*) (1·25 g.), m. p. 125—170° (total yield of acidic material 87%).

Grateful acknowledgment is made to the Medical Research Council for an assistantship (for J. C. R.), to the Rockefeller Foundation, the Government Grants Committee of the Royal Society, and Imperial Chemical Industries Ltd. for generous grants, and to Mr. Z. M. Zochowski for his help with some of the synthetic work.

THE UNIVERSITY, ST. ANDREWS.
THE UNIVERSITY, BRISTOL.

[*Received, September 17th,* 1954.]