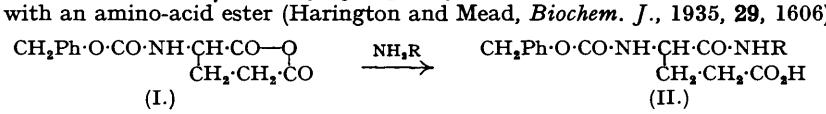
399. *Amino-acids and Peptides. Part I. An Examination of the Use of Carbobenzyloxy-L-glutamic Anhydride in the Synthesis of Glutamyl-peptides.*

By W. J. Le Quesne and G. T. Young.

In the preparation of α-L-glutamyl amides, peptides, and esters by the ring opening of *N*-carbobenzyloxy-L-glutamic anhydride, appreciable amounts of the γ-isomers may also be formed. With hydrazine, a mixture of α- and γ-acid hydrazides has been obtained. Reaction of the anhydride with the appropriate amino-acid esters yielded carbobenzyloxy-α-L-glutamyl-L-valine ethyl ester, carbobenzyloxy-α-L-glutamyl-L-leucine ethyl ester, and diethyl carbobenzyloxy-α-L-glutamyl-L-aspartate; in each case purification was effected by fractional extraction with aqueous sodium carbonate. Hydrolysis and hydrogenation then yielded the corresponding dipeptides.

After reaction of the anhydride with ethanol, this method of purification enabled crystalline α-ethyl carbobenzyloxy-L-glutamate to be isolated, which on hydrogenation gave α-ethyl L-glutamate; evidence is given that the compounds previously ascribed these structures are in fact racemic. α-Benzyl carbobenzyloxy-L-glutamate has now been obtained crystalline.

The classical synthesis of α-glutamyl peptides depends on the reaction of *N*-carbobenzyloxy L-glutamic anhydride (I) with amino-acid esters, followed by hydrolysis and hydrogenation (Bergmann and Zervas, *Ber.*, 1932, **65**, 1192). In analogous fashion, ammonia reacts with the anhydride to give carbobenzyloxy-L-*iso*glutamine (II; R = H), which on hydrogenation yields L-*iso*glutamine. The anhydride ring may also be opened with alcohols or with alkoxides, and the α-esters so formed have been used for the synthesis of γ-glutamyl-peptides such as glutathione, since the free γ-carboxyl group may be converted into the acid chloride and so coupled with an amino-acid ester (Harington and Mead, *Biochem. J.*, 1935, **29**, 1606).



(I.) (II.)

There have, however, been indications that the anhydride ring may not always open exclusively in this direction. With glycylglycine ethyl ester, Bergman, Zervas, and Fruton

(*J. Biol. Chem.*, 1936, **115**, 606) obtained by repeated recrystallisation only a small yield of the α-derivative and concluded that a considerable portion of the γ-isomer had been formed. Melville (*Biochem. J.*, 1935, **29**, 179) examined the reaction with ammonia, and estimated that the crude L-*iso*glutamine contained some 14% of L-glutamine. More recently, Boothe *et al.* (*J. Amer. Chem. Soc.*, 1949, **71**, 2310) separated both of the possible isomers from the reaction of the anhydride with triethyl α-L-glutamyl-L-glutamate. Similar anomalies occur with the analogous carbobenzyloxy-L-aspartic anhydride; from the reaction with glycine ethyl ester the α-derivative was obtained (Bergmann, Zervas, and Fruton, *J. Biol. Chem.*, 1935, **111**, 235), but with tyrosine ethyl ester the β-compound was isolated (Bergmann, Zervas, Salzmann, and Schleich, *Z. physiol. Chem.*, 1934, **224**, 17). Investigations in the aspartic acid series will form the subject of a later communication. Other acyl-L-glutamic anhydrides appear to react irregularly; Nicolet (*J. Amer. Chem. Soc.*, 1930, **52**, 1192) has stated that acetyl-L-glutamic anhydride yields the γ-amide with ammonia and with aniline, and King and Kidd (*J.*, 1949, 3315) have found that phthaloyl-L-glutamic anhydride also gives γ-derivatives.

It seemed possible that the ambiguity of the anhydride route might be avoided, if hydrazine were to react with carbobenzyloxy-L-glutamic anhydride to yield an acid hydrazide of determinable structure; a preliminary note of the results of this investigation has appeared (Le Quesne and Young, *Nature*, 1949, **163**, 604). With aqueous hydrazine at 0°, a crystalline hydrazide was obtained in good yield. The melting points of successive preparations were reasonably sharp but varied between 166° and 171°, being unchanged in each case by recrystallisation; the benzylidene derivatives also varied in melting point. Conversion into the azide and coupling with glycine ethyl ester gave a product from which a small quantity of carbobenzyloxy-α-L-glutamylglycine ethyl ester was isolated, but hydrolysis and hydrogenation of the coupling product gave a mixture of α- and γ-glutamyl-peptides since with ninhydrin 0·8 mole of carbon dioxide was evolved (Van Slyke, Dillon, MacFadyen, and Hamilton, *J. Biol. Chem.*, 1941, **141**, 627). Curtius degradation of the azide in benzyl alcohol gave γγ-dicarbobenzyloxyaminobutyric acid (which on hydrogenation yielded β-formylpropionic acid, identified as the *p*-nitrophenylhydrazone), but hydrogenation of the ether-soluble portion of the degradation products gave L-αγ-diaminobutyric acid, characterised as the dipicrate. It is clear therefore that the carbobenzyloxy-L-glutamylhydrazide was a mixture of the α- and the γ-isomer, which repeated crystallisation failed to separate. Authentic samples of both isomers have since been prepared (Part II; Le Quesne and Young, following paper) and from their known specific rotations it appears that the samples prepared by the anhydride route varied greatly in composition, containing between 8 and 88% of the α-form. Reaction in chloroform solution gave similar mixtures.

It seems likely that in ring openings of this type we must expect the formation of both isomers; crystallisation of the main product may thus be hindered, whilst in some cases mixed crystals may be formed. We have therefore developed a method which has proved useful for the purification of certain of such products. The acid strength of carbethoxyglycine (pK' 3·65) is considerably greater than that of acetic acid (pK 4·76) and it is therefore to be expected that coupling products with free α-carboxyl groups will be more acidic than their γ-isomers. Fractional extraction with aqueous sodium carbonate of solutions of such materials in organic solvents has in fact been found of great value. In this way it has been found possible to isolate a small sample of carbobenzyloxy-γ-L-glutamylglycine ethyl ester from the reaction of the anhydride (I) with glycine ethyl ester.

With this procedure, we have examined the reaction of carbobenzyloxy-L-glutamic anhydride with the ethyl esters of L-valine, L-leucine, and L-aspartic acid. For the coupling we have used a modification of the method of Boothe *et al.* (*loc. cit.*); the ester hydrochloride was dissolved in aqueous potassium hydrogen carbonate in contact with a layer of ethyl acetate, and the anhydride in ethyl acetate solution was added gradually with stirring. In this way we obtained carbobenzyloxy-α-L-glutamyl-L-valine ethyl ester, which on hydrolysis yielded carbobenzyloxy-α-L-glutamyl-L-valine; hydrogenation gave α-L-glutamyl-L-valine. Analogous series of reactions yielded α-L-glutamyl-L-leucine and -L-aspartic acid. Whilst exploring this method of coupling, we prepared also by this route the previously known ethyl esters of carbobenzyloxy-α-L-glutamyl-glycine, -L-glutamic acid, and -glycylglycine. The yields were somewhat lower (*ca.* 40% calculated on the anhydride) than normal, but the economy in the use of ester is considerable. In the last case the yield was only 21%, but this is perhaps to be expected since the conditions would permit some cyclisation to diketopiperazine.

For the preparation of carbobenzyloxy-L-aspartic anhydride, Miller, Behrens, and du Vigneaud (*J. Biol. Chem.*, 1941, **140**, 411) found it advantageous to close the ring by the use

of acetic anhydride at room temperature. We find that similarly mild conditions, followed by vacuum-distillation of the solvent at 50—60°, lead to carbobenzyloxy-L-glutamic anhydride of slightly increased optical activity ($[\alpha]_D$ $-45{\cdot}1° \pm 0{\cdot}2°$ in glacial acetic acid; Bergmann and Zervas, *loc. cit.*, reported $[\alpha]_D$ $-44{\cdot}1°$).

We then extended our investigation of the ring opening of carbobenzyloxy-L-glutamic anhydride to the reaction with alcohols. α-Methyl carbobenzyloxy-L-glutamate has been obtained as a syrup by reaction with sodium methoxide (Harington and Mead, *loc. cit.*); with sodium ethoxide Melville (Thesis, London, 1934) obtained an oil which he considered to be α-ethyl carbobenzyloxy-L-glutamate admixed with some 30% of the γ-isomer. By treating the anhydride with ethanol at 125°, Neuberger (*Biochem. J.*, 1936, **30**, 2085) obtained a syrup which slowly crystallised, yielding a product, m. p. 100°, which appeared to be the α-ester. Since then, other workers have made unsuccessful attempts to repeat this preparation (see, *e.g.*, Boothe *et al.*, *J. Amer. Chem. Soc.*, 1948, **70**, 1099). Despite many variations in conditions we also failed to obtain this compound, but by fractional extraction of the reaction products with aqueous carbonate we isolated instead a substance, m. p. 46—48°, which with ammonia yielded carbobenzyloxy-L-*iso*glutamine and with hydrazine gave carbobenzyloxy-α-L-glutamylhydrazide (see Le Quesne and Young, following paper). Hydrogenation gave material, m. p. 115—117°, compared with 110° reported by Neuberger for his α-ethyl L-glutamate. It appeared possible that it was a case of polymorphism, and through the kindness of Professor A. C. Chibnall and Dr. Neuberger we obtained a small specimen of the higher-melting material prepared by Neuberger's method at Cambridge. A mixture of the two melted over a considerable range and interconversion could not be achieved. Finally, we prepared carbobenzyloxy-DL-glutamic anhydride, which in hot ethanol yielded a crystalline product shown by its properties to be α-ethyl carbobenzyloxy-DL-glutamate, m. p. 99—101°; mixed with the Cambridge sample (of m. p. 95—97°), the m. p. was 95—99°. For comparison we prepared γ-ethyl carbobenzyloxy-DL-glutamate from γ-ethyl-DL-glutamate; it had m. p. 83—85°, depressed on admixture with the isomer. The ease of crystallisation and low solubility of the racemic α-derivative would explain its isolation from syrups in which it is a minor constituent. We consider therefore the compounds reported by Neuberger to be racemic and we place on record our constants for α-ethyl carbobenzyloxy-L-glutamate and α-ethyl-L-glutamate.

α-Benzyl carbobenzyloxy-L-glutamate has previously been prepared as an oil, by treating the anhydride with benzyl alcohol at 100° (Bergmann, Zervas, and Salzmann, *Ber.*, 1933, **66**, 1288). By fractional extraction with aqueous sodium carbonate of a solution of the reaction products in ether, we have now obtained this useful intermediate as a crystalline solid, m. p. 78—81°.

Experimental.

All m. p.s are uncorrected. Combustion analyses are by Drs. Weiler and Strauss and by Mr. F. C. Hall.

Reaction between Carbobenzyloxy-L-glutamic Anhydride and Hydrazine.—(a) *In aqueous solution.* Carbobenzyloxy-L-glutamic anhydride (12 g.) was dissolved portion-wise with stirring at 0° in aqueous hydrazine hydrate (30%; 48 ml.). The solution was acidified to Congo-red with concentrated hydrochloric acid, and then saturated aqueous sodium acetate was added until it was no longer acid to Congo-red. After 30 minutes, the precipitate was filtered off and washed with water. Recrystallisation from boiling water (250 ml.) gave mixed crystals (*A*) of carbobenzyloxy-α- and -γ-L-glutamylhydrazides (11·25 g., 84%), m. p. 166—167° raised by recrystallisation to 168° (Found: C, 52·6; H, 5·7; N, 14·5. Calc. for $C_{13}H_{17}O_5N_3$: C, 52·9; H, 5·8; N, 14·2%), $[\alpha]_D^{21}$ −17·9° (*c*, 6·19 in 0·5N-hydrochloric acid); other samples had $[\alpha]_D$ varying between −14·2° and −22·2°.

(b) *In chloroform solution.* A solution of carbobenzyloxy-L-glutamic anhydride (5 g.) in chloroform (25 ml.) was added gradually with shaking to a mixture of hydrazine (anhydrous; 1·7 ml.) and chloroform (25 ml.) at 0°. After some minutes, the solution was extracted with water (10 ml.) twice and the combined aqueous extracts were washed with ether. The aqueous layer was acidified to Congo-red with concentrated hydrochloric acid, filtered, and neutralised with saturated aqueous sodium acetate. It was set aside at 0° for 30 minutes and the mixed crystals were filtered off (2·85 g., 64%), m. p. 154—158°, raised by recrystallisation to 163—166°, $[\alpha]_D^{18}$ −19·0° (*c*, 7·9 in 0·5N-hydrochloric acid).

Benzylidene Derivative of Product A.—Carbobenzyloxy-L-glutamylhydrazide (*A*) (0·1 g.) was dissolved in aqueous acetic acid (50%; 10 ml.), and benzaldehyde (0·1 ml.) added. After 30 minutes at 0°, the precipitate was filtered off, washed with water and ether, and air-dried; it had m. p. 197°, unaltered by recrystallisation from aqueous ethanol (Found: C, 62·8; H, 5·6; N, 11·1. Calc. for $C_{20}H_{21}O_5N_3$: C, 62·6; H, 5·5; N, 11·0%). Different samples of hydrazide gave products melting between 179° and 203°.

Conversion of Product A *into the Azide, and Coupling with Glycine Ethyl Ester.*—A solution of carbobenzyloxy-L-glutamylhydrazide (*A*) (2·8 g.) in a mixture of water (60 ml.) and concentrated hydrochloric acid (3·5 ml.) was covered with chloroform (30 ml.) and stirred mechanically at 0°. A solution of sodium nitrite (1·1 g.) in water (5 ml.) was added portion-wise during 5 minutes and stirring continued for an additional 10 minutes. The chloroform layer was then separated, washed twice with ice-cold water, and dried (Na_2SO_4) for a few seconds. It was then slowly added at 0° to a solution of glycine

ethyl ester (from 12 g. of hydrochloride) in chloroform (75 ml.) and left for 30 minutes in the ice-bath. After 5 hours at room temperature it was washed with dilute hydrochloric acid and water, dried (Na_2SO_4), and evaporated under reduced pressure. The product (*B*) (2·05 g., 59%) crystallised under light petroleum; it had m. p. 80—90°, raised by repeated recrystallisation from ethyl acetate–light petroleum to 96—104°.

To the solution obtained by extraction of part of this material with boiling ether, light petroleum was added. After the mixture had been kept at 0° overnight, a little carbobenzyloxy-α-L-glutamylglycine ethyl ester was filtered off; it had m. p. 120—123°, and mixed with the ring-opening product had m. p. 119—121°.

L-*Glutamylglycine from Coupling Product* B.—Carbobenzyloxy-L-glutamylglycine ethyl ester (mixture of α- and γ-compounds, Product *B* above) (0·45 g.) was dissolved in N-potassium hydroxide (2·5 ml.) and left for 30 minutes at room temperature. The solution was then acidified with 5N-hydrochloric acid and extracted twice with ethyl acetate; the combined extracts were washed with dilute hydrochloric acid and water, dried (Na_2SO_4), and evaporated under reduced pressure. The remaining oil was dissolved in aqueous methanol and hydrogenated in the presence of palladium black at atmospheric pressure. The filtrate obtained was evaporated to small volume under diminished pressure and excess of ethanol added. The L-glutamylglycine obtained by filtration gave 0·8 mole of carbon dioxide with ninhydrin at 100° and pH 2·5.

Curtius Degradation of Product A.—A solution of carbobenzyloxy-L-glutamylhydrazide (A) (2 g.) in a mixture of water (50 ml.) and concentrated hydrochloric acid (2·5 ml.) was covered with a layer of ether (50 ml.). With mechanical stirring at 0°, an aqueous solution of sodium nitrite (0·8 g.) was added portion-wise during 5 minutes, then stirring continued for another 10 minutes. The ethereal layer was washed twice with ice-cold water, dried (Na_2SO_4) for a few minutes at 0°, and filtered into redistilled benzyl alcohol (6 ml.). The ether was removed under reduced pressure at room temperature, then the solution slowly heated to 100° and kept at that temperature for 2 hours. After cooling, ether (20 ml.) and light petroleum (b. p. 60—80°; 100 ml.) were added. The oil which separated was triturated with ether, a white solid remaining; the ethereal extract, evaporated *in vacuo*, gave an oil (*C*). The solid *γγ-dicarbobenzyloxyaminobutyric acid* (0·3 g., 12%) had m. p. 168—171°, raised by recrystallisation from aqueous ethanol to 173—174° (Found: C, 62·3; H, 5·5; N, 7·2. $C_{20}H_{22}O_6N_2$ requires C, 62·2; H, 5·7; N, 7·3%).

β-Formylpropionic Acid.—A solution of γγ-dicarbobenzyloxyaminobutyric acid in aqueous methanol containing a drop of acetic acid and some palladium black was hydrogenated at normal pressure. The solution obtained after filtration reduced Fehling's solution and ammoniacal silver nitrate. The methanol was removed under reduced pressure and the remaining solution treated with *p*-nitrophenylhydrazine in dilute hydrochloric acid, giving a yellow precipitate. This was recrystallised from hot water giving golden plates of the β-formylpropionic acid *p*-nitrophenylhydrazone, m. p. 174—177° (Found: C, 50·9; H, 4·7; N, 17·9. Calc. for $C_{10}H_{11}O_4N_3$: C, 50·6; H, 4·7; N, 17·7%).

L-*αγ-Diaminobutyric Acid.*—The ether-soluble oil (*C*) from the Curtius degradation was hydrogenated in aqueous methanol acidified with acetic acid, palladium black being used as catalyst. The solution was filtered and evaporated to small volume under reduced pressure. Saturated aqueous picric acid (40 ml.) was added, and the solution left at 0° overnight. Yellow crystals of the dipicrate of L-αγ-diaminobutyric acid were obtained, having m. p. 180—182° (Found: C, 33·1; H, 3·1; N, 19·3. Calc. for $C_{16}H_{16}O_{16}N_8$: C, 33·3; H, 2·8; N, 19·5%).

Carbobenzyloxy-L-glutamic Anhydride.—Carbobenzyloxy-L-glutamic acid (20 g.) and acetic anhydride (50 ml.) were set aside with occasional shaking until complete dissolution occurred. The solvent was removed *in vacuo* at 50—60° and the residue dissolved in dry chloroform (40 ml.) and ether (40 ml.) by warming. After 1 hour at 0°, the carbobenzyloxy-L-glutamic anhydride was filtered off (13·0 g., 70%), m. p. 93—94°, $[\alpha]_D$ −45·1° (*c*, 9·98 in glacial acetic acid).

L-*Valine Ethyl Ester Hydrochloride.*—L-Valine (2 g.) and ethanol (25 ml.) saturated with hydrogen chloride were refluxed together for 3 hours. After removal of the solvent under reduced pressure, the *ester hydrochloride* crystallised when kept in a vacuum-desiccator; recrystallisation from ethyl acetate–light petroleum gave deliquescent crystals (2·2 g., 71%), m. p. 93—97° in a sealed tube (Found: Cl, 19·9. $C_7H_{16}O_2NCl$ requires Cl, 19·5%).

Carbobenzyloxy-α-L-glutamyl-L-valine Ethyl Ester.—A solution of L-valine ethyl ester hydrochloride (1·1 g.) and potassium hydrogen carbonate (1·5 g.) in water (10 ml.), covered with ethyl acetate (10 ml.), was stirred mechanically while a solution of carbobenzyloxy-L-glutamic anhydride (1·5 g.) in ethyl acetate (10 ml.) was added portion-wise during 2—3 minutes; stirring was continued for 2 hours. Next morning the aqueous layer was separated, acidified with 5N-hydrochloric acid and extracted twice with ethyl acetate. After being washed with water, the ethyl acetate layer was successively extracted with aqueous sodium carbonate (four portions each containing 0·06 g., then 0·5 g.) and the separate extracts acidified with 5N-hydrochloric acid. The oil which separated from the last fraction soon crystallised, and repetition of the fractional extraction procedure on the oil obtained from the penultimate extraction gave more solid material. Recrystallisation from ethyl acetate gave *carbobenzyloxy-α-L-glutamyl-L-valine ethyl ester* (1·0 g., 32%), m. p. 119—121°, raised to 122—124° by further recrystallisation (Found: N, 7·0. $C_{20}H_{28}O_7N_2$ requires N, 6·9%).

Carbobenzyloxy-α-L-glutamyl-L-valine.—Carbobenzyloxy-α-L-glutamyl-L-valine ethyl ester (0·8 g.) in N-sodium hydroxide (5 ml.) was left for 1 hour at room temperature. Acidification with 5N-hydrochloric acid gave an oil which soon crystallised. After 30 minutes, the *carbobenzyloxy-α-L-glutamyl-L-valine* was filtered off, washed with water, air-dried, and washed with boiling ether. The product (0·5 g., 68%) had m. p. 130—132°, raised to 131—133° by recrystallisation from ethyl acetate–light petroleum (Found: C, 56·8; H, 6·4; N, 7·8. $C_{18}H_{24}O_7N_2$ requires C, 56·8; H, 6·4; N, 7·4%).

α-L-*Glutamyl-L-valine.*—Carbobenzyloxy-α-L-glutamyl-L-valine (0·7 g.) was hydrogenated in the usual way in aqueous methanol. α-L-*Glutamyl-L-valine* separated during the reduction and, after filtration, was extracted with boiling water and reprecipitated with ethanol as shiny plates (0·45 g.), m. p. 182—183°, raised by recrystallisation from aqueous ethanol to 189—190° (Found: C, 48·9; H,

7·4; N, 10·8. $C_{10}H_{18}O_5N_2$ requires C, 48·8; H, 7·4; N, 11·4%), $[\alpha]_D^{15}$ +24·5° (*c*, 2·65 in water containing 1 mole of hydrochloric acid).

Carbobenzyloxy-α-L-glutamyl-L-leucine Ethyl Ester.—A solution of L-leucine ethyl ester hydrochloride (1·25 g.; m. p. 127—129°; from commercial L-leucine) and potassium hydrogen carbonate (2 g.) in water (15 ml.), covered with ethyl acetate (10 ml.), was treated with carbobenzyloxy-L-glutamic anhydride (1·65 g.) in ethyl acetate (10 ml.) as for the valine ester. The final fraction from the sodium carbonate treatment was extracted into ethyl acetate, dried (Na_2SO_4), and evaporated under reduced pressure. The product slowly crystallised under light petroleum, and was recrystallised from toluene (3—4 ml.), giving *carbobenzyloxy-α-L-glutamyl-L-leucine ethyl ester* (0·8 g., 30%), m. p. 88—94°, raised to 94—96° by recrystallisation from ethyl acetate–light petroleum (Found: C, 59·5; H, 7·0; N, 6·8. $C_{21}H_{30}O_7N_2$ requires C, 59·7; H, 7·2; N, 6·6%).

α-L-Glutamyl-L-leucine.—Carbobenzyloxy-α-L-glutamyl-L-leucine ethyl ester (0·7 g.) was dissolved in N-sodium hydroxide (5·5 ml.) and left at room temperature for 1 hour. The solution was acidified with 5N-hydrochloric acid and extracted 3 times with ethyl acetate. The combined extracts were washed with a little water, dried (Na_2SO_4), and evaporated under reduced pressure, leaving carbobenzyloxy-α-L-glutamyl-L-leucine as a syrup. This was hydrogenated in the usual way in methanolic solution. Reduction took about 12 hours. The solution was filtered and the precipitate washed with boiling water. The combined filtrates were evaporated under reduced pressure to small volume, and ethanol was added. After 1 hour at 0°, the *α-L-glutamyl-L-leucine* was filtered off and washed with ethanol. It forms shiny plates, m. p. 196—197° (Found: C, 50·8; H, 7·5; N, 10·8. $C_{11}H_{20}O_5N_2$ requires C, 50·7; H, 7·7; N, 10·8%), $[\alpha]_D^{18}$ +8·6° (*c*, 1·75 in water containing 1 mole of hydrochloric acid).

Diethyl Carbobenzyloxy-α-L-glutamyl-L-aspartate.—Diethyl L-aspartate hydrochloride (3·6 g.; recrystallised from ethanol–ether; m. p. 98—102° in a sealed tube) and potassium hydrogen carbonate (4·0 g.) were dissolved in water (40 ml.), and ethyl acetate was added (20 ml.). The mixture was treated with a solution of carbobenzyloxy-L-glutamic anhydride (3·6 g.) in ethyl acetate (20 ml.) as for the valine ester. The last fraction and that obtained by refractionation of previous fractions with sodium carbonate gave, on acidification, oils which rapidly crystallised to give *diethyl carbobenzyloxy-α-L-glutamyl-L-aspartate* (2·6 g., 43%), m. p. 107—118°, raised to 118—120° by recrystallisation from ethyl acetate–light petroleum (Found: C, 55·6; H, 6·3; N, 6·0. $C_{21}H_{28}O_9N_2$ requires C, 55·8; H, 6·2; N, 6·2%).

Carbobenzyloxy-α-L-glutamyl-L-aspartic Acid.—Diethyl carbobenzyloxy-α-L-glutamyl-L-aspartate (1·5 g.) was dissolved in N-sodium hydroxide (11 ml.) and left at room temperature for 40 minutes. After being acidified with 5N-hydrochloric acid and kept for 30 minutes, *carbobenzyloxy-α-L-glutamyl-L-aspartic acid* (1·1 g., 79%) was filtered off, washed with a little water, and dried. The m. p. was 170—173°, raised by recrystallisation from hot water to 171—174° (Found: C, 51·2; H, 5·2; N, 6·8. $C_{17}H_{20}O_9N_2$ requires C, 51·5; H, 5·1; N, 7·1%).

α-L-Glutamyl-L-aspartic Acid.—Carbobenzyloxy-α-L-glutamyl-L-aspartic acid was hydrogenated in the usual manner in aqueous methanol. Evaporation of the filtrate and washings to small volume, and precipitation with ethanol, gave *α-L-glutamyl-L-aspartic acid monohydrate*, m. p. 138—141° (Found: C, 38·6; H, 5·7; N, 9·9. $C_9H_{14}O_7N_2,H_2O$ requires C, 38·6; H, 5·8; N, 10·0%), $[\alpha]_D^{18}$ +30·2° (*c*, 2·32 in water).

Diethyl Carbobenzyloxy-α-L-glutamyl-L-glutamate.—Diethyl L-glutamate hydrochloride (3 g.) was dissolved in aqueous potassium hydrogen carbonate covered with ethyl acetate, and treated with carbobenzyloxy-L-glutamic anhydride (3 g.) in a similar manner to the above esters. Diethyl carbobenzyloxy-α-L-glutamyl-L-glutamate was obtained (2·3 g., 43%), m. p. 132—134°.

Carbobenzyloxy-α- and -γ-glutamylglycine Ethyl Esters.—Glycine ethyl ester hydrochloride (2·0 g.) was treated similarly with potassium hydrogen carbonate and carbobenzyloxy-L-glutamic anhydride (3·0 g.). Carbobenzyloxy-α-L-glutamylglycine ethyl ester (1·6 g., 38%), m. p. 120—123°, was obtained from the final sodium carbonate extract. Refractionation of the first extract gave a small quantity of *carbobenzyloxy-γ-L-glutamylglycine ethyl ester*, m. p. 105—108°, unchanged by admixture with the γ-compound prepared as in the subsequent paper.

Carbobenzyloxy-α-L-glutamylglycylglycine Ethyl Ester.—Glycylglycine ethyl ester hydrochloride (1·5 g.) in aqueous potassium hydrogen carbonate covered with ethyl acetate was treated with carbobenzyloxy-L-glutamic anhydride (1·8 g.) in ethyl acetate as above. From the last fractions obtained by sodium carbonate extraction, carbobenzyloxy-α-L-glutamylglycylglycine ethyl ester was isolated (0·5 g., 21%), m. p. 134—136°.

α-L-Glutamylglycylglycine.—Carbobenzyloxy-α-L-glutamylglycylglycine was hydrogenated in the usual manner in aqueous methanol. Evaporation of the filtrate to low bulk and addition of ethanol gave *α-L-glutamylglycylglycine*, m. p. 160—162° (Found: C, 41·1; H, 5·5. $C_9H_{15}O_6N_3$ requires C, 41·4; H, 5·8%).

Paper Partition Chromatography of Some α-Glutamyl-peptides.—By use of phenol saturated with water in an atmosphere containing ammonia, the following R_F values were obtained, each peptide giving a single spot with ninhydrin: α-L-glutamyl- 0·15, -L-tyrosine 0·36, -L-valine 0·42, -L-leucine 0·59, -L-glutamic acid 0·06, -L-aspartic acid 0·04, and -glycylglycine 0·19 (glycine, 0·43; glutamic acid, 0·18).

α-Ethyl Carbobenzyloxy-L-glutamate.—Carbobenzyloxy-L-glutamic anhydride (14 g.) was heated in a sealed tube with ethanol (70 ml.) at 120—130° for 4 hours. After cooling, the excess of ethanol was removed under reduced pressure, and the residue dissolved in ether. The solution was extracted with 4 successive portions of aqueous sodium carbonate (each containing 0·6 g.), then with excess. The final fraction on acidification gave an oil which rapidly solidified. Refractionation of the other extracts gave more *α-ethyl carbobenzyloxy-L-glutamate monohydrate* (total: 5·1 g., 29%), m. p. 46—48° (Found: C, 55·4; H, 6·8. $C_{15}H_{19}O_6N,H_2O$ requires C, 55·0; H, 6·5%). Drying over phosphoric oxide in a vacuum-desiccator gave the anhydrous *ester*, m. p. 46—48° (Found: C, 58·6; H, 6·3; N, 4·7. $C_{15}H_{19}O_6N$ requires C, 58·2; H, 6·2; N, 4·5%), $[\alpha]_D^{16}$ −21·4° (*c*, 7·7 in ethanol). The mixed m. p. with Prof. Chibnall's sample (m. p. 95—97°) was 41—90°.

Refractionation of the first sodium carbonate extracts gave γ-ethyl carbobenzyloxy-L-glutamate,

which after recrystallisation from ethyl acetate–light petroleum, had m. p. 82—84°. The mixed m. p. with the authentic γ-compound was 81—84°.

*Carbobenzyloxy-*DL*-glutamic Anhydride.*—Carbobenzyloxy-DL-glutamic acid (6·5 g.; m. p. 122—124°) was treated as the L-compound above, giving *carbobenzyloxy-*DL*-glutamic anhydride* (5·6 g., 90%), m. p. 108—109° (Found: C, 59·2; H, 5·2; N, 5·5. $C_{13}H_{13}O_5N$ requires C, 59·3; H, 5·0; N, 5·3%).

*α-Ethyl Carbobenzyloxy-*DL*-glutamate.*—Carbobenzyloxy-DL-glutamic anhydride (2 g.) was treated as for the L-compound. The final fractions from sodium carbonate extraction on acidification gave oils which rapidly solidified. The *α-ethyl carbobenzyloxy-*DL*-glutamate* was washed with boiling ether, and another crop was obtained by cooling the filtrate (total: 0·85 g., 35%). The m. p. was 99—101° (Found: C, 58·1; H, 6·2. $C_{15}H_{19}O_6N$ requires C, 58·2; H, 6·2%). The mixed m. p. with Prof. Chibnall's sample (m. p. 95—97°) was 95—99°.

*γ-Ethyl Carbobenzyloxy-*DL*-glutamate.*—γ-Ethyl DL-glutamate (1·6 g.) was treated in the same manner as the L-compound (Abderhalden and Nienburg, *Z. physiol. Chem.*, 1933, **219**, 155), giving *γ-ethyl carbobenzyloxy-*DL*-glutamate* (1·8 g., 64%), m. p. 82—85°, raised by recrystallisation from ether–light petroleum to 83—85°(Found: C, 58·6; H, 5·9. $C_{15}H_{19}O_6N$ requires C, 58·2; H, 6·2%). The mixed m. p. with the α-compound was 70—77°.

α-Ethyl L*-Glutamate.*—α-Ethyl carbobenzyloxy-L-glutamate was hydrogenated in the usual manner in ethanolic solution. The filtrate was evaporated to small volume and ether added. The α-ethyl L-glutamate was filtered off and reprecipitated from ethanolic solution with ether, then having m. p. 116—118°. Drying over phosphoric oxide in a vacuum-desiccator at room temperature did not remove all the solvent; heating to 100° caused decomposition.

*Carbobenzyloxy-*L*-iso**glutamine.*—α-Ethyl carbobenzyloxy-L-glutamate (1 g.) was dissolved in aqueous ammonia (*d* 0·880; 10 ml.) and left for 4 days. The solution was acidified with concentrated hydrochloric acid, with cooling, and filtered after 1 hour. The carbobenzyloxy-L-*iso*glutamine was recrystallised from hot water, with m. p. 168—172° (0·7 g., 75%). The mixed m. p. with a sample prepared by Bergmann's method was 167—172°.

*α-Benzyl Carbobenzyloxy-*L*-glutamate.*—Carbobenzyloxy-L-glutamic anhydride (3 g.) and benzyl alcohol (1·5 g.) were heated at 100° for 3½ hours, cooled, and dissolved in ether. The solution was fractionally extracted with aqueous sodium carbonate. The last fraction was acidified and extracted with ether. After the ethereal layer had been dried and the solvent removed under reduced pressure, the product solidified during some days. It was washed with cold toluene (2—3 ml.) and the α-benzyl carbobenzyloxy-L-glutamate filtered off (0·8 g., 18%), having m. p. 75—80°. It was dissolved in a little ethanol, precipitated by adding water, and filtered off after being kept overnight. It was air-dried and washed with a little cold toluene, to give m. p. 78—81° (Found: C, 64·5; H, 5·6; N, 4·0. Calc. for $C_{20}H_{21}O_6N$: C, 64·8; H, 5·7; N, 3·8%).

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