693. *A New Synthesis of Glutamine and of γ-Dipeptides of Glutamic Acid from Phthalylated Intermediates.*

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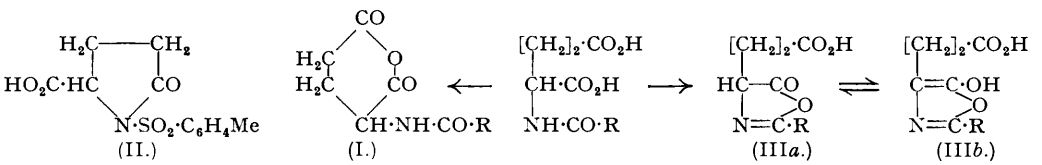
Condensation of phthalic anhydride with L-glutamic acid gives *phthalyl-DL-glutamic acid*; in order to obtain the corresponding L-*acid* it is necessary to proceed *via ethyl* o-*carboxybenzoyl*-L-*glutamate*. *Phthalyl*-DL- and -L-*glutamic anhydrides* differ from carbobenzyloxyglutamic anhydride in combining with ammonia to form the *γ-amides* in high yields. As already briefly reported (*Nature*, 1948, **162**, 776), hydrolysis of the phthalyl group with hydrazine at room temperature affords a direct synthesis of DL-*glutamine* and of the naturally occurring L-isomer. The dipeptides γ-DL-*glutamylglycine* and γ-L-glutamyl-L-glutamic acid have also been prepared by this method.

THE fundamental biological significance of glutamic acid, already well recognised from its occurrence as glutamine and as a constituent of glutathione and of a variety of proteins, has lately been strikingly emphasised by the discovery of the folic acid series of growth factors. There are indications, for example, in Woolley's investigations on strepogenin (*J. Biol. Chem.*, 1948, **172**, 71), of other important developments in the chemistry of glutamic acid, and the synthesis of its simple peptides has thus become a matter of considerable interest. Owing to the peculiar difficulties arising from the presence of the second carboxyl group, the problem is one not readily solved by the classical methods of peptide synthesis due to Fischer, and the preparation of glutamylamino-acids, *e.g.*, the glutamylglutamic acids, was not successfully accomplished until the introduction of the carbobenzyloxy-synthesis by Bergmann and Zervas (*Ber.*, 1932, **65**, 1192).

The unique feature of Bergmann's method is its use of the acylglutamic anhydride (I; R = O·CH_2Ph) as an acylating agent, the peptide link being formed simultaneously with the opening of the ring. Only carbobenzyloxyglutamic acid has so far been applied in this way, largely because most other protecting substituents cannot be eliminated without destroying the peptide bonds. The toluene-*p*-sulphonyl group, which should be free from this difficulty, cannot apparently be used owing to the preferential dehydration of toluene-*p*-sulphonyl-glutamic acid to a derivative of the pyrrolidonecarboxylic acid (II) (Harington and Moggridge,

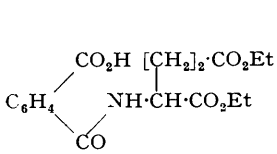


J., 1940, 706). A further complication which may arise in the employment of carboxylic acids for the protection of the glutamic amino-group is that when dissolved in acetic anhydride the α-acylamido-acids, with the exception of the carbonato-compounds, are very readily dehydrated to oxazolones. Although these products may be utilised in the synthesis of α-glutamyl derivatives (see, *e.g.*, King and Spensley, forthcoming publication), the optically active glutamic acid residue is very likely to suffer racemisation—according to du Vigneaud and Meyer (*J. Biol. Chem.*, 1932, **99**, 143) as a result of tautomeric changes, such as (III*a*) ⇌ (III*b*).

A hitherto uninvestigated route to the glutamylamino-acids and peptides has lately been successfully completed through the utilisation of compounds protected by the phthalyl group

(Kidd and King, *Nature*, 1948, **162**, 776). Unpublished experiments (1936—1939) by Sir Robert Robinson and the senior author, in which a series of simple phthalylated peptides of glycine and alanine was prepared, have shown that the phthalyl group can easily be removed with hydrazine leaving the peptide chain unaffected. In addition to the highly crystalline character of the intermediates, the method carries with it the advantage that, owing to the tertiary character of the nitrogen atom in the phthalimido-compounds, oxazolone formation cannot interfere with attempts to obtain the phthalylglutamic anhydride.

Phthalimido-acids are commonly prepared by heating mixtures of the amino-acids and phthalic anhydride slightly above the fusion point of the anhydride, but the product so obtained from L-glutamic acid was not obtained pure when crystallised from water. The condensation was then attempted in presence of solvents, *e.g.*, acetic acid, pyridine, and, in order to ensure cyclisation of the initially-formed phthalamic acid, the solvent was evaporated and the residue warmed with acetic anhydride. As expected, glutamic anhydride ring-closure also occurred, but was accompanied by racemisation, presumably at the phthalamic acid stage, the product being *phthalyl-*DL*-glutamic anhydride* (IV), which on dissolution in hot water gave



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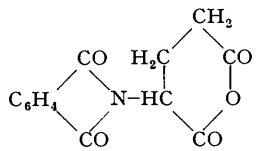

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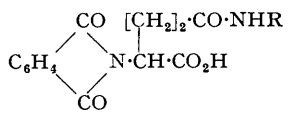

(VI.)

*phthalyl-*DL*-glutamic acid*. Racemisation was avoided by carrying out the synthesis under milder conditions using ethyl L-glutamate in place of the free amino-acid. The required ester was obtained from its hydrochloride by neutralisation with anhydrous diethylamine, a method already applied to the preparation of other amino-esters by Harington and Mead (*Biochem. J.*, 1935, **29**, 1602), and on treatment of an ethereal solution with phthalic anhydride *ethyl o-carboxybenzoyl-*L*-glutamate* (V) was rapidly formed. Ring-closure to *ethyl phthalyl-*L*-glutamate* was effected either by refluxing of (V) with ethanolic hydrogen chloride and distillation, or by reaction with cold thionyl chloride followed by heating of the mixture in benzene. *Phthalyl-*L*-glutamic acid* was then obtained by hydrolysis with a boiling mixture of acetic and hydrochloric acids.

During the course of this work, Billman and Harting (*J. Amer. Chem. Soc.*, 1948, **70**, 1473) reported the preparation of the phthalyl-L-acid by heating L-glutamic acid with phthalic anhydride at 180°. The melting point of their compound was almost identical with that of our phthalyl-DL-glutamic acid, and it was confirmed by repetition of their experiment, that their synthesis had led to racemisation.

The reactions of the phthalylglutamic anhydrides were investigated first with the DL-isomer, which was dissolved in dioxan and treated with ethereal ammonia. The product obtained on acidification of the precipitated ammonium salt was subjected to degradation with sodium hypobromite and, after hydrolysis of the phthalyl group with boiling hydrochloric acid, precipitation with phosphotungstate, etc., DL-$\alpha\gamma$-diaminobutyric acid was isolated as the picrate. By this series of reactions, which follows Karrer's work on asparagine and glutamine (*Helv. Chim. Acta*, 1923, **6**, 411, 957; 1926, **9**, 301), it was established that the phthalylglutamic anhydride had given *phthalyl-*DL*-glutamine*, *i.e.*, the γ-amide (VI; R = H). The exclusive formation of a γ-derivative (isolated in 74% yield) is perhaps without parallel in the series of acylglutamic anhydrides. Carbobenzyloxylglutamic anhydride, though sometimes affording mixtures contain low proportions of γ-amides (Melville, *Biochem. J.*, 1935, **29**, 179; LeQuesne and Young, *Nature*, 1949, **163**, 604), generally gives the α-compounds. Nicolet (*J. Amer. Chem. Soc.*, 1930, **52**, 1192) has recorded the preparation of γ-amides from acetylglutamic anhydride, but the latter was not purified and the constitution of the products, which were not isolated, rests solely on the formation, with ammonium thiocyanate and acetic anhydride, of derivatives having the composition of acetylthiohydantoins.

For the hydrolysis of the phthalyl group in the phthalimido-acids and peptides the conditions used by Ing and Manske (*J.*, 1926, 2348), *i.e.*, heating with hydrazine in boiling alcohol, were considered too drastic. Sheehan and Frank (*J. Amer. Chem. Soc.*, 1949, **71**, 1856) in some experiments on the synthesis of peptides communicated after the appearance of our earlier publication (Kidd and King, *loc. cit.*) have successfully used the original Ing and Manske method; in view of the presence of a carboxyl group, however, the phthalylated compounds

can be dissolved in aqueous sodium carbonate, whereupon the addition of 1 equivalent of hydrazine detaches the phthalyl residue *in the cold*. The resulting phthalhydrazide is in due course precipitated by neutralisation with 2N-hydrochloric acid, and the solution then contains the amino-acid or peptide together with sodium chloride. Where difficulty occurs in their separation, hydriodic acid can be used for neutralisation, the solubility of sodium iodide in cold alcohol, in which the peptide is invariably sparingly soluble, enabling the inorganic material to be removed. In this way phthalyl-DL-glutamine was hydrolysed to DL-*glutamine*, the presence of the γ-amido-group in the product being confirmed by the van Slyke determination.

Crystalline derivatives were also obtained from phthalyl-DL-glutamic anhydride on treatment with aniline, benzylamine, or glycine, and by analogy with the ammonia reaction product these are *phthalyl-γ-DL-glutamylaniline* (VI; R = Ph), *-γ-DL-glutamylbenzylamine* (VI; R = CH_2Ph), and *-γ-glutamylglycine* (VI; R = $CH_2{\cdot}CO_2H$). Hydrolysis of the last-named compound with hydrazine yielded DL-*γ-glutamylglycine*, its constitution as a γ-amide being confirmed by the Van Slyke ninhydrin determination (*J. Biol. Chem.*, 1941, **141**, 627), the liberation of carbon dioxide being equivalent to one free α-amino-acid residue.

The optically active *anhydride* was obtained by the action of acetic anhydride on phthalyl-L-glutamic acid, thus confirming the conclusion that racemisation is the result of oxazolone formation. From the L-anhydride and ammonia, *phthalyl-L-glutamine* (77%) was obtained, its constitution being demonstrated by hydrolysis with hydrazine to L-glutamine, identical in properties with the natural compound and liberating 96—98% of its amino-nitrogen in the Van Slyke determination.

When phthalyl-L-glutamic anhydride and ethyl L-glutamate were set aside in dioxan–ether at room temperature *ethyl phthalyl-γ-L-glutamyl-L-glutamate* was formed. Hydrolysis with cold N-alkali and then hydrazine gave glutamyl-γ-glutamic acid which, when *p*-nitrobenzoylated in the form of its ethyl ester, subsequently afforded *p*-nitrobenzoylglutamyl-γ-glutamic acid, identical with that synthesised by Boothe *et al.* (*J. Amer. Chem. Soc.*, 1948, **70**, 1099) through the carbobenzyloxy-dipeptide.

Experimental.

Phthalyl-DL-glutamic Anhydride.—A suspension of L-glutamic acid (32·6 g.) and phthalic anhydride (32·6 g.) in dry pyridine (120 c.c.) was refluxed for 2 hours. After evaporation of the clear solution under reduced pressure, acetic anhydride (90 c.c.) was added and the mixture boiled for 2—3 minutes. Crystallisation of the product began during concentration of the solvent at diminished pressure and was completed, on cooling, by the cautious addition of anhydrous ether. After being washed with ether and dried, the *phthalyl-DL-glutamic anhydride* (41 g., 74%) had m. p. 195—196° (decomp.), unchanged by recrystallisation from ethyl acetate from which it separated in colourless stout prisms (Found: C, 60·6; H, 3·6; N, 5·8. $C_{13}H_9O_5N$ requires C, 60·2; H, 3·5; N, 5·4%).

Phthalyl-DL-glutamic Acid.—(*a*) The phthalylglutamic anhydride was dissolved in the minimum quantity of boiling water; on cooling, *phthalyl-DL-glutamic acid* crystallised in colourless needles, m. p. 189—190° (Found: C, 56·5; H, 4·0; N, 5·0. $C_{13}H_{11}O_6N$ requires C, 56·3; H, 4·0; N, 5·1%).

(*b*) Equimolecular quantities of L-glutamic acid and of phthalic anhydride heated at 180°, as described by Billman and Harting (*loc. cit.*), and then treated with water gave a product with feeble optical activity, but on recrystallisation the DL-acid, m. p. and mixed m. p. 189—190°, was obtained. At 130—140° the reaction appeared to be incomplete, the product not being purifiable by recrystallisation from water.

Phthalyl-DL-glutamine.—A solution of phthalyl-DL-glutamic anhydride (5 g.) in warm dioxan (40 c.c.) was cooled in water and treated with small quantities of dry ethereal ammonia in excess. When precipitation was complete, the bulky ammonium salt was collected, washed with ether, and dissolved in a little water. On acidification (Congo-red) with 5N-hydrochloric acid, *phthalyl-DL-glutamine* (3·9 g., 74%) slowly separated, and after several hours in the refrigerator was collected and recrystallised from water, giving small colourless prisms, m. p. 194—195° (Found: C, 56·0; H, 4·2; N, 10·0. $C_{13}H_{12}O_5N_2$ requires C, 56·5; H, 4·4; N, 10·1%).

DL-*Glutamine.*—A solution of the phthalylglutamine (5·4 g.) in aqueous sodium carbonate (1·35 g. in 25 c.c.) was treated with hydrazine hydrate (2 g. of 50%) and set aside for 2 days at room temperature. Hydrochloric acid (2N.) was then added, the precipitate of phthalhydrazide removed, and chloride ion eliminated by shaking the solution with silver carbonate (7·5 g.) for several hours. After filtration the clear liquid was exactly neutralised with 2N-hydriodic acid and concentrated at 30° under reduced pressure. The addition of alcohol then precipitated DL-*glutamine*, which by crystallisation from aqueous acetone was obtained as colourless glistening prisms, m. p. 185—186° (Found: C, 41·0; H, 6·85; N, 19·4. $C_5H_{10}O_3N_2$ requires C, 41·1; H, 6·85; N, 19·2%). With Nessler's reagent a colour appeared gradually after 3 minutes. In the Van Slyke determination 2 moles of nitrogen were liberated.

DL-αγ-*Diaminobutyric Acid.*—Phthalyl-DL-glutamine (3·5 g.) was dissolved in aqueous sodium hypobromite prepared from bromine (2·35 g.) and sodium hydroxide (2·6 g.) in water (45 c.c.), and the solution heated at 70—80° for 1 hour. After cooling, the mixture was acidified with 5N-hydrochloric acid, filtered, and concentrated to 20 c.c., and, after further addition of hydrochloric acid (25 c.c. of 35%), the solution was heated under reflux for 3 hours. Phthalic acid was then removed from the diluted solution by filtration and ether-extraction, and the diaminobutyric acid precipitated as phosphotungstate. This was dissolved in aqueous acetone and treated with barium hydroxide, and, after

removal of the barium phosphotungstate and precipitation of excess barium as sulphate, the filtrate was evaporated to small bulk under reduced pressure. On admixture of the product with aqueous picric acid αγ-diaminobutyric acid picrate separated, and recrystallisation gave long yellow prisms, m. p. 184—185° (cf. Carter, Abeele and Rothrock, *J. Biol. Chem.*, 1949, **178**, 325) (Found : C, 33·6; H, 2·6; N, 19·4. Calc. for $C_4H_{10}O_2N_2,2C_6H_3O_7N_3$: C, 33·3; H, 2·8; N, 19·55%).

*Phthalyl-γ-*DL*-glutamylaniline.*—Phthalyl-DL-glutamic anhydride (1·5 g.), suspended in ether (20 c.c.), was treated with aniline (1·5 g.), and the mixture shaken at room temperature for 30 minutes. The *anilide* (1·8 g., 86%) was collected and washed with ether, and when recrystallised from water formed colourless prisms of the hydrate, m. p. 106—107° (Found : C, 62·0; H, 4·8; N, 7·6. $C_{19}H_{16}O_5N_2,H_2O$ requires C, 61·6; H, 4·9; N, 7·6%).

*Phthalyl-γ-*DL*-glutamylbenzylamine.*—Benzylamine (4·2 g., 1 mol.) was added to a supercooled solution of the phthalylglutamic anhydride (10 g., 1 mol.) in dioxan (20 c.c.), and the mixture set aside at room temperature for 30 minutes. Dilution with anhydrous ether precipitated an oil which solidified when stirred under ether. The *benzylamine* (10·3 g., 72%) crystallised from dioxan–ether in small prisms, or from aqueous alcohol in tiny plates, m. p. 196—198°, raised after 2 further crystallisations to 203—204° (Found : C, 65·3; H, 5·0; N, 7·3. $C_{20}H_{18}O_5N_2$ requires C, 65·6; H, 4·9; N, 7·7%).

*Phthalyl-γ-*DL*-glutamylglycine.*—Phthalylglutamic anhydride (20 g.) was dissolved in hot acetic acid (150 c.c.) containing glycine (5·8 g.) and the solution set aside for 20 minutes. It was then evaporated at low pressure, leaving a straw-coloured gum which slowly solidified. Recrystallisation from water gave *phthalyl-γ-*DL*-glutamylglycine* (12 g., 48%) in hard masses of minute prisms, m. p. 200—201° raised to 201—202° by further crystallisation (Found : C, 53·7; H, 4·3; N, 8·1. $C_{15}H_{14}O_7N_2$ requires C, 53·9; H, 4·2; N, 8·4%).

*γ-*DL*-Glutamylglycine.*—The phthalyl-dipeptide (4·3 g., 1 mol.) was dissolved in water (60 c.c.) with the addition of sodium carbonate (1·43 g., 2·1 mols.) and then of 60% hydrazine hydrate (1·1 g., 1 mol.). After 2 days, the solution was made acid to Congo-red with 10% hydriodic acid, and treated with a little sodium acetate. Later, the solution was filtered from phthalhydrazide and evaporated to dryness at low pressure. The residue was submitted to azeotropic drying with absolute ethanol and anhydrous benzene, and when triturated with alcohol formed a hygroscopic crystalline powder. After 2 recrystallisations from ethanol containing traces of water, *γ-*DL*-glutamylglycine* was obtained as a non-hygroscopic microcrystalline solid, m. p. 178—179° (Found : C, 40·3; H, 5·9; N, 13·1. $C_7H_{12}O_5N_2$ requires C, 40·8; H, 5·8; N, 13·6%). In the ninhydrin determination, 0·0371 g. of the dipeptide gave carbon dioxide equivalent to 0·00256 g. of α-amino-nitrogen; calc., 0·00255 g.

Ethyl o*-Carboxybenzoyl-*L*-glutamate.*—The crystalline ester hydrochloride obtained from L-glutamic acid (28·5 g., 1 mol.) and boiling 4% ethanolic hydrogen chloride (400 c.c.) was treated under benzene with diethylamine (24·4 c.c., 1·2 mols.) added in portions with cooling. After thorough mixing, diethylamine hydrochloride was precipitated with anhydrous ether and removed by filtration; ethyl L-glutamate (32·2 g., 82%) was obtained by evaporation of the solvents.

To an ethereal solution of the ester, phthalic anhydride (23·5 g., 1 mol.) was added in small quantities, which when shaken dissolved with evolution of heat. Scratching or seeding caused the separation of *ethyl* o*-carboxybenzoyl-*L*-glutamate* (46·2 g., 84%) as a solid mass which was collected after 24 hours and washed with ether. Crystallisation from benzene–light petroleum gave a flocculent precipitate of the pure ester, m. p. 94° (Found : C, 57·6; H, 6·0; N, 4·2. $C_{17}H_{21}O_7N$ requires C, 58·1; H, 6·0; N, 4·0%).

*Ethyl Phthalyl-*L*-glutamate.*—(*a*) Ethyl *o*-carboxybenzoyl-L-glutamate (0·7 g.) was heated in refluxing 4% ethanolic hydrogen chloride (10 c.c.) for 2 hours. The product remaining on evaporation was shaken with water and chloroform, and the chloroform layer washed with aqueous sodium hydrogen carbonate and water, dried, and evaporated. The residue of *ethyl phthalyl-*L*-glutamate* distilled (bath temperature, 190—200°) at 0·05 mm. as a colourless viscous syrup, η^{22} 1·5220, $[\alpha]_D^{19}$ −33·5° in ethanol (Found : N, 4·1. $C_{17}H_{19}O_6N$ requires N, 4·2%).

(*b*) Ethyl *o*-carboxybenzoyl-L-glutamate (46·2 g.) was set aside with purified thionyl chloride (90 c.c.) at room temperature, and, when the reaction was over, the clear solution was evaporated and benzene distilled from the residue on a water-bath at 70°. The product, after being washed in ethereal solution with sodium hydrogen carbonate solution and water, was dried and evaporated as before, the pure phthalyl-L-glutamic ester, η^{21} 1·5224, $[\alpha]_D^{20}$ −33·2° in ethanol, distilling at 0·14 mm. (bath temperature, 190°) (Found : C, 61·5; H, 5·9; N, 3·9. $C_{17}H_{19}O_6N$ requires C, 61·3; H, 5·7; N, 4·2%).

*Phthalyl-*L*-glutamic Acid.*—The undistilled ester (50·5 g.) was dissolved in a mixture of acetic acid (450 c.c.) and concentrated hydrochloric acid (110 c.c.), and the solution heated under reflux for 2 hours. Evaporation to 100 c.c. at reduced pressure and storage for several hours in the refrigerator caused *phthalyl-*L*-glutamic acid*, m. p. 158—159°, to separate, which on crystallisation from water formed small colourless prisms (28·2 g., 65%), m. p. 158—159°, $[\alpha]_D^{18}$ −27·4° in 0·33N-sodium carbonate (Found : C. 56·5; H, 4·0; N, 4·9. $C_{13}H_{11}O_6N$ requires C, 56·3; H, 4·0; N, 5·0%).

*Phthalyl-*L*-glutamic Anhydride.*—Phthalyl-L-glutamic acid (7·5 g.) was dissolved in acetic anhydride (20 c.c.) and heated on a steam-bath for 5 minutes. The *anhydride* (4·65, 67%) was isolated, by concentration of the solution under reduced pressure and addition of ether, as stout prisms melting at 195—196° to a turbid liquid which cleared at 200°. From ethyl acetate it separated in solvated highly-refracting prisms, m. p. 190—191°, which when dried at 140° under low pressure crumbled to powder having the original m. p. of 195—200° (Found, on the dried material : C, 60·3; H, 3·6; N, 5·6. $C_{13}H_9O_5N$ requires C, 60·2; H, 3·5; N, 5·4%). Dissolution in hot water gave phthalyl-L-glutamic acid of unchanged m. p. and optical rotation.

*Phthalyl-*L*-glutamine.*—The action of ammonia on phthalyl-L-glutamic anhydride (4·9 g.) under conditions used for the DL-compound gave *phthalyl-*L*-glutamine* (4 g., 77%), m. p. 160—162°, which on recrystallisation from water gave colourless needles, m. p. 163°, $[\alpha]_D^{18}$ −16·9° in 0·33N-sodium carbonate (Found : C, 56·7; H, 4·6; N, 9·7. $C_{13}H_{12}O_5N_2$ requires C, 56·5; H, 4·4; N, 10·1%).

L*-Glutamine.*—Removal of the phthalyl group of phthalyl-L-glutamine was effected as in the hydrolysis of the comparable DL-compound. The resulting L-glutamine (yield 67%) was recrystallised by rendering its aqueous solution turbid with ethanol and then storage in the refrigerator. The product separated

as tiny needles, m. p. 182—183° (decomp.), $[\alpha]_D^{22}$ 5·6° in water (Found: C, 41·0; H, 7·0; N, 18·4. Calc. for $C_5H_{10}O_3N_2$: C, 41·1; H, 6·9; N, 19·2%). With Nessler's reagent it slowly gave a colour after 5 minutes and in the Van Slyke determination 96—98% of the total nitrogen was liberated.

Ethyl Phthalyl-L-γ-glutamyl-L-glutamate.—Solutions of ethyl L-glutamate (5·6 g.) in ether and of phthalyl-L-glutamic anhydride (3·5 g.) in dioxan were mixed and set aside at room temperature for 30 hours. After evaporation of the solvents under reduced pressure and addition of ethyl acetate the product crystallised, and recrystallisation from aqueous ethanol gave the *phthalyl-dipeptide ethyl* ester as lustrous prisms, m. p. 162° (Found: C, 57·0; H, 5·4; N, 6·1. $C_{22}H_{26}O_9N_2$ requires C, 57·1; H, 5·6; N, 6·1%).

p-*Nitrobenzoyl-γ-L-glutamyl-L-glutamic Acid.*—The phthalyl-γ-L-glutamyl-L-glutamic ester (2 g.) was treated with N-sodium hydroxide (5 c.c.) and a few drops of thymolphthalein solution. Further small amounts of N-alkali (total, 2 mols.) were slowly introduced to maintain the blue colour of the solution as the hydrolysis proceeded. Finally, hydrazine hydrate (4 c.c. of 9%) was added and the mixture set aside for 36 hours. Phthalhydrazide was precipitated by 2N-hydrochloric acid and, after filtration, the solution was evaporated to dryness and esterified with saturated ethanolic hydrogen chloride (25 c.c.) at room temperature. The dipeptide ester hydrochloride obtained on evaporation was converted into the *p*-nitrobenzoyl derivative according to the directions of Boothe *et al.* (*loc. cit.*), the recrystallised acid separating in stout needles, m. p. 195° (Found: C, 47·8; H, 4·3; N, 9·4. Calc. for $C_{17}H_{19}O_{10}N_3$: C, 48·0; H, 4·5; N, 9·9%).

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