CCXXVII. SYNTHESIS OF PEPTIDES CONTAINING CYSTINE AND GLUTAMINE, WITH REMARKS ON THEIR POSSIBLE BEARING ON THE STRUCTURE OF INSULIN AND A NOTE ON THE AMIDE NITROGEN OF INSULIN.

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WHILST it is universally accepted that insulin, as isolated from the pancreas, is a protein, the possibility is not yet excluded that this protein may owe its physiological activity to a specific grouping. The suggestion was made by Harington & Scott [1929] that an analogy might exist between insulin and thyroglobulin which is physiologically active by virtue of its content of thyroxine, although it was recognized by these authors that the separation of the active constituent from the whole protein which can be effected in the case of thyroglobulin might prove quite impracticable in that of insulin.

All attempts which have been made to separate an active substance of low molecular weight from insulin have in fact proved fruitless, since the mildest degradation of the molecule, by proteolytic enzymes or by other methods, has invariably resulted in total loss of physiological activity. In spite of these failures, however, the impression that insulin does indeed possess an active "prosthetic" group has persisted and the evidence bearing on such an assumption has been summarized by Jensen & Evans [1934].

From the numerous experiments which have been made on the physiological inactivation of insulin by treatment with mild hydrolytic agents two main conclusions have emerged: (1) a significant proportion of the nitrogen of insulin is liberated as ammonia under conditions which suggest that it occurs in the protein as a labile amide and such loss of ammonia involves loss of activity; (2) the sulphur of insulin is very labile towards alkali and treatment with alkali sufficient to cause elimination of even a part of the sulphur also inactivates insulin. Lability of amide nitrogen in proteins is generally associated with the occurrence therein of glutamine and the sulphur of cystine is well known to become highly labile when the amino-acid occurs in certain types of peptide linkage. It is known from other sources that insulin yields on hydrolysis a large proportion both of glutamic acid and cystine, and Jensen & Evans [1934] were thus led to suggest that the hypothetical active group of insulin might contain cystine and glutamine in peptide linkage with one another.

Until quite recently it has been impossible to prepare peptides containing cystine and glutamine; within the last two or three years, however, methods have become available [Bergmann & Zervas, 1932; Bergmann *et al.*, 1933; du Vigneaud *et al.*, 1930; Siffert & du Vigneaud, 1935; Harington & Mead, 1935] which offered some prospect of success and it was therefore considered worth while to attempt the synthesis of cysteylglutamine and glutaminyleysteine in order to subject the suggestion of Jensen & Evans to direct test.

Whilst it was recognized that the possibility that either of these simple compounds would itself exhibit hypoglycaemic activity was remote it was hoped that a study of their respective chemical behaviours in relation to that of insulin might afford definite evidence in favour of or against the occurrence of a similar grouping in the hormone.

Synthetical experiments.

The syntheses, which follow accepted lines and are briefly described below, call for no detailed comment. Two general points in connection with them may however be mentioned. The first concerns the preparation of cystine derivatives and the second the use of benzyl esters in peptide synthesis. It was found while this work was in progress that even the simpler derivatives of cystine are very difficult to crystallize. Two crystalline compounds of this amino-acid are described in the appendix to the experimental section but these were but two successes among many failures. When the syntheses of cystylglutamine and glutaminylcystine were first undertaken, the route chosen passed through a series of cystine compounds and although both peptides were eventually obtained as crystals in their reduced forms, no success attended many attempts to crystallize several of the intermediates. When, however, cysteine derivatives were substituted for those of cystine no difficulties were encountered. It was necessary at times to protect the sulphhydryl group and here the use of *S*-benzyl derivatives, which has been introduced by du Vigneaud, was found to be very satisfactory.

It will be recalled that the method of peptide synthesis devised by Bergmann & Zervas [1932] depends upon the removal of *N*-benzylcarbonyl- and of *O*-benzyl-groups by means of a reduction process. It was observed during the present work that removal of a benzyl ester group by reduction with phosphonium iodide proceeds very much more slowly than the removal of an *N*-benzylcarbonyl-group under identical conditions. One experiment with an *S*-benzyl-compound indicated that removal of this group probably proceeds more slowly still. These differences in ease of reduction may be of use in synthetic work. An example occurs in the preparation of cysteine benzyl ester hydroiodide (p 1605).

Cysteylglutamine. *N*-Benzylcarbonyl-*S*-benzylcysteine (I), prepared in the usual manner from *S*-benzylcysteine and benzylcarbonyl chloride, was converted into the acid chloride and coupled in aqueous solution in presence of triethylamine with γ -monoethyl glutamate to give γ -monoethyl-*N*-benzylcarbonyl-*S*-benzylcysteylglutamate (II); the latter was treated with ammonia and the resulting amide was freed from benzyl and benzylcarbonyl residues by reduction with sodium in liquid ammonia to yield cysteylglutamine (III).

I

II

III

Glutaminylcysteine. *N*-Benzylcarbonylcystine benzyl ester, prepared by the action of *N*-benzylcarbonylcystyl chloride on benzyl alcohol was converted into cysteine benzyl ester hydroiodide by partial reduction with phosphonium iodide; the cysteine benzyl ester was then coupled with *N*-benzylcarbonylglutamic anhydride to yield *N*-benzylcarbonyl- α -glutamylcysteine benzyl ester (IV); the *S*-benzyl derivative of the latter was converted in turn into the acid chloride and

the amide (V) which was finally reduced with sodium in liquid ammonia to give glutaminylcysteine (VI).

Physiological tests.

We are indebted to Dr H. P. Himsworth for testing the two synthetic peptides for hypoglycaemic activity. The peptides were tested as such and after oxidation to the disulphide forms by means of hydrogen peroxide. In each case the material under test (10 mg.) was dissolved in sterile saline (2.5 ml.) and injected into a rabbit, the blood sugar being determined at intervals for a period of 1 hour. No suggestion of hypoglycaemic activity was obtained. It may be noted that glycylcystine, cystylglycine and γ -glutamylcystine have recently been found to be devoid of physiological activity [Jensen *et al.*, 1936].

Labile sulphur.

Table I gives the proportions of labile sulphur in insulin, glutathione and the new synthetic peptides together with some values from the literature. The substances were boiled in 0.1 *N* sodium carbonate solution during 45 min. and the

Table I.

Substance	"Sodium carbonate-labile sulphur" (%)
Glutaminylcystine	8.2
Cystylglutamine	17.8
Glutathione (oxidized)	37.3
Insulin (crystalline)	60.0
Cystine*	2.8
Alanylcystine*	18.6
Alanylcystine anhydride*	91.8
Insulin†	37.4

* Brand & Sandberg [1926].

† Abel *et al.* [1927].

figures are percentages of the total sulphur eliminated as hydrogen sulphide upon acidification and boiling after the treatment with carbonate. The conditions were standardized as far as possible but appear to be more severe than those used by earlier workers since our result for insulin is considerably higher than that previously reported.

It will be seen that cystylglutamine and glutaminylcystine resemble alanylcystine and are intermediate in their behaviour between cystine itself and the more complex glutathione. Insulin is much less stable.

Liberation of ammonia during treatment with acid and alkali.

The peptides were treated with 0.1 *N* HCl and with *N*/30 NaOH under the conditions prescribed for insulin by Jensen & Evans [1932]. The values for insulin are taken from these authors. The results are presented as percentages of the total amide nitrogen.

The liberation of ammonia from insulin in acid was measured under conditions in which the substance would be present as the insoluble "heat precipitate".

Table II. *Percentage of nitrogen liberated as ammonia by treatment with acid and alkali.*

Substance	Treatment	
	N/10 HCl at 100° 1 hour	N/30 NaOH at 34° 3 hours
Glutaminyleystine	100	9.4
Cystylglutamine	69.5	5.7
Insulin [Jensen & Evans, 1932]	(23)	10.9

A comparison therefore of the result so obtained with those from the (soluble) peptides would have little significance.

Whereas the sulphur of cystylglutamine is more labile than that of glutaminyleystine in the case of the amide nitrogen the position is reversed. If the doubtful result obtained by acid treatment of insulin is neglected it appears that the lability of the amide nitrogen in insulin is little different from that in the peptides; the sulphur of insulin on the other hand is far more labile than that of either of the peptides. In so far as deductions can be made from the study of the simple peptides the properties of the latter therefore afford no support to the suggestion that the cystylglutamine or glutaminyleystine linkage occurs in the molecule of insulin. This conclusion must be accepted with reserve however since the lability of the sulphur in such peptides may well be increased by attachment of additional amino-acid residues.

Amide nitrogen and glutamic acid content of insulin.

Glutamic acid has been isolated from insulin by Jensen & Wintersteiner [1932]. Since these workers were unable to detect the presence of aspartic acid, it may be assumed provisionally that glutamic acid is the only dicarboxylic acid present. It may also be assumed that this acid is in combination with the peptide structure of the molecule through its α -amino- and α -carboxyl-groups only, the γ -carboxyl-group either remaining "free" or in combination as an (acid) amide. As the total number of free acidic groups in the insulin molecule is now known with some accuracy [Harington & Neuberger, 1936] it follows that an estimate of the amount of glutamic acid present may be made if the amide nitrogen content is also known. It should be pointed out that this estimate will be subject to two sources of error. First it is not certain that all free acidic groups are revealed by titration and secondly a small part of the base binding is attributable to terminal carboxyl groups. These errors will affect the result in opposite directions.

Our present knowledge of the amide nitrogen content of insulin is based on the amount of ammonia liberated during complete hydrolysis [Wintersteiner *et al.*, 1928]. It has been shown by Shore *et al.* [1935] however that, in the case of some proteins at least, ammonia is liberated during hydrolysis from sources other than amide groups. It therefore appeared desirable to determine the amide nitrogen of insulin by the method described by these workers.

Experiments on the hydrolysis of insulin by 0.2 N HCl at 100° were unsatisfactory because the substance remained undissolved even after 20 hours. Determinations were therefore made of the ammonia liberated by the treatment of insulin with 5N HCl for varying periods of time. The results which are given in Fig. 1 lead to a value of approximately 34 equivalents of amide nitrogen per molecule assuming the molecular weight of insulin to be 35,100. The quantities of insulin employed and therefore the accuracy of the results were severely limited by the expensive nature of the material. It is hoped however that sufficient accuracy has been achieved to make possible a rough estimate of the

amount of glutamic acid in insulin. If the number of free acidic groups due to glutamic acid is assumed to be roughly 32, it follows that the proportion of this acid in insulin is about 30 %. This figure is of course subject to the limitations mentioned above.

Fig. 1. Liberation of ammonia from insulin during treatment with 5*N* HCl at 100°.

EXPERIMENTAL.

Synthesis of cysteylglutamine.

S-Benzylcysteine. Cystine was reduced by means of zinc dust and HCl to cysteine which was isolated as its cuprous mercaptide. The latter was decomposed with H₂S and the solution after removal of cuprous sulphide was concentrated, made alkaline and treated with benzyl bromide in the usual manner. The product was recrystallized from water and formed large elongated plates or prisms which melted at 219° with decomposition after sintering at 205°. The yield was 58 % of the theoretical.

It is probable that this compound may be prepared more conveniently by the method of du Vigneaud *et al.* [1930].

N-Benzylcarbonyl-S-benzylcysteine. *S*-Benzylcysteine (5.0 g.) in 2*N* NaOH (12 ml.) was treated with benzylcarbonyl chloride (5 ml.; 25 % excess) and 2*N* NaOH (15 ml.) with vigorous shaking and ice cooling during 30 min. The mixture was finally shaken mechanically for a further 30 min. During the course of the reaction the sodium salt of the product separated as a heavy layer. The whole was washed three times with ether. The latter was discarded and the two heavier layers were made acid to Congo red by the addition of 5*N* HCl and were extracted three times with ethyl acetate. The ethyl acetate solution was washed well with saturated NaCl, dried over sodium sulphate, concentrated to a small volume under diminished pressure and treated cautiously with ligroin. Crystallization started at once and was completed by the addition of ligroin and by standing at 0°. The substance was collected and recrystallized from benzene. The yield was 90 % of the theoretical. *N*-Benzylcarbonyl-*S*-benzylcysteine forms colourless elongated prisms melting at 93–95° after sintering. (Found: N, 4.17; S, 9.10 %. C₁₈H₁₉O₄NS requires N, 4.06; S, 9.28 %. 42.5 mg. required 6.10 ml. *N*/50 NaOH for neutralization to cresol red. C₁₈H₁₉O₄NS titrating as a monobasic acid requires 6.16 ml.)

N-Benzylcarbonyl-*S*-benzylcysteine was also prepared as follows: *N*-benzylcarbonylcystine in 80 % alcoholic sulphuric acid (0.75 *N*) was reduced with zinc-copper couple to *N*-benzylcarbonylcysteine which formed prismatic needles (m.p. 82°) from ethyl acetate-ligroin. *N*-Benzylcarbonylcysteine was treated with benzyl bromide in alkaline solution giving *N*-benzylcarbonyl-*S*-benzylcysteine in 63 % yield.

γ-Monoethyl-*N*-benzylcarbonyl-*S*-benzylcysteylglutamate. *N*-Benzylcarbonyl-*S*-benzylcysteine (4.0 g.) was suspended in dry ether (30 ml.). The mixture was cooled in ice-salt and shaken with phosphorus pentachloride (2.8 g.; 15 % excess). The undissolved material soon passed into solution and after about 5 min. nearly all the phosphorus pentachloride had disappeared. The pale yellow solution was decanted from excess reagent and evaporated to a syrup at a low temperature and with exclusion of atmospheric moisture. The residue was treated with light petroleum (b.p. 60–80°) and soon crystallized in small prisms. The acid chloride was collected, washed with light petroleum and added slowly to a mechanically stirred ice-cold solution of *γ*-ethylglutamate (3.0 g.; 50 % excess) and triethylamine (4.0 ml.) in water (15 ml.). When rapid stirring had been maintained for 10 min. triethylamine (1.0 ml.) and a small quantity of ether were added and any lumps were broken up with a rod. After being stirred during a further 10 min. the mixture was centrifuged. The upper layer was discarded and the aqueous layer was decanted from a small residue, acidified and extracted three times with ethyl acetate. The ethyl acetate solution was washed with dilute HCl and several times with saturated NaCl. It was dried over sodium sulphate in the presence of norite, concentrated under diminished pressure to a small volume and treated cautiously with ligroin. The oil which separated crystallized after a short period at 0°. The material after collection weighed 3.6 g. or 62 % of the theoretical amount. It was recrystallized from ethyl acetate and formed fine needles which melted at 88° after sintering at 85°. (Found: N, 5.47; S, 6.42 %. $C_{25}H_{31}O_7N_2S$ requires N, 5.58; S, 6.37 %). 49.7 mg. in methyl alcohol required 5.01 *N*/50 NaOH for neutralization to cresol red. $C_{25}H_{31}O_7N_2S$ titrating as a monobasic acid requires 4.95 ml.

N-Benzylcarbonyl-*S*-benzylcysteylglutamine. *γ*-Monoethyl-*N*-benzylcarbonyl-*S*-benzylcysteylglutamate (7.3 g. of recrystallized material) was dissolved in concentrated ammonia solution (140 ml. of sp. gr. 0.880). After standing in a warm place overnight the solution was centrifuged in order to remove a trace of flocculent material and was concentrated under diminished pressure until excess of ammonia had been removed. The residue was diluted to 100 ml. with water and treated with a slight excess of 2 *N* HCl. A white viscous oil separated which hardened rapidly. It was collected and crystallized from *n*-propyl alcohol. The yield was 5.3 g. or 78 % of the theoretical. After a second crystallization from *n*-propyl alcohol the substance formed fine needles which melted at 188–189° after sintering at 186°. (Found: N, 8.8; S, 6.31 %. $C_{23}H_{27}O_6N_3S$ requires N, 8.9; S, 6.76 %.)

Cysteylglutamine. *N*-Benzylcarbonyl-*S*-benzylcysteylglutamine (3.6 g.) was dissolved in liquid ammonia (35 ml.) in an apparatus fitted with a mechanical stirrer. The solution was treated gradually with sodium in small pieces (0.7 g.: 4 equiv.) and was cooled in an acetone-CO₂ mixture until nearly all the sodium had been added. When the blue colour due to excess sodium had become permanent the bulk of the ammonia was allowed to evaporate and the last traces were removed by suction. The residue was suspended in dry alcohol (15 ml.) cooled to –30° and treated with sufficient HI to give a faintly acid solution. The latter was diluted with water, extracted with ether in order to remove dibenzyl

and then treated cautiously with 5*N* ammonia until the *pH* had risen to about 5.0. The solution was then evaporated to a small volume under diminished pressure and treated rapidly with alcohol. The granular precipitate was collected and washed with alcohol at the centrifuge until the washings no longer gave a test for iodide. The crude peptide was finally collected. After being dried over phosphoric oxide *in vacuo* it weighed 1.4 g. or 74 % of the theoretical amount. Attempts to crystallize the crude peptide were unsuccessful.

The substance in portions of 200 mg. was dissolved in water (3.0 ml.). The solution was treated with 10 drops of 0.5 *N* H₂SO₄ and then with a suspension of cuprous oxide first in the cold and then with gentle warmth until a small dark red deposit appeared. The solution generally began to darken at this point. The cuprous compound which was grey-violet in colour and which dissolved in water to form a clear violet solution, separated when the solution was cooled. Precipitation was completed by adding 3 vol. of alcohol. The precipitate was suspended in a small volume of water containing 3 drops of 0.5 *N* H₂SO₄ and was then treated with alcohol. The precipitates from all the 200 mg. portions were united at this stage and were washed 10 times at the centrifuge by suspension in water followed by treatment with alcohol. The material was finally suspended in water and decomposed with H₂S. The cuprous sulphide was removed and washed with water at the centrifuge. The solution and washings were concentrated to a small volume under diminished pressure and adjusted to *pH* 4.8 by the addition of 5*N* ammonia. Crystallization started at once and continued during further evaporation of the solution over phosphoric oxide *in vacuo*. The product (290 mg.) was collected, washed with water, 50 % alcohol and absolute alcohol and was dried at 56° *in vacuo*. A second crop (422 mg.) of less pure material was obtained by allowing the mother liquor to stand over alcohol in a desiccator at a pressure of 300 mm.

It may be of interest to record that when the mother liquors from the preparation of the violet-coloured cuprous mercaptide were worked up by way of the compound formed with mercuric sulphate, the peptide recovered from the latter derivative gave a white cuprous mercaptide which was not appreciably soluble in water.

Cysteylglutamine forms colourless plates or prisms, and is more soluble in water than the isomeric glutaminylcysteine. (Found: C, 38.2; H, 6.26; N, 16.6; S, 12.40; amide N, 5.5, 5.56 %. C₈H₁₅O₄N₃S requires: C, 38.6; H, 6.02; N, 16.9; S, 12.85; amide N 5.6 %.)

Optical rotation. Cysteylglutamine. 15.1 mg. in 0.5 ml. water had $\alpha_{5461} + 0.1^\circ$ in a 0.5 dm. tube whence $[\alpha]_{5461} = +6.6^\circ$.

Cystylglutamine. 10.3 mg. of the peptide in 0.797 ml. of water containing the theoretical amount of hydrogen peroxide and a trace of copper sulphate had $\alpha_{5461} - 0.09^\circ$ in a 0.5 dm. tube when oxidation was complete. $[\alpha]_{5461} = -13.9^\circ$.

Iodine titration. The peptide (9.8 mg.) was dissolved in 7.5 % potassium iodide solution (5 ml.) and treated with approximately 0.01 *N* iodine (5.0 ml.). The excess of iodine was back-titrated with 0.01 *N* sodium thiosulphate. 3.81 ml. of 0.01 *N* iodine were required, the theoretical quantity being 3.94 ml. of 0.01 *N*.

Synthesis of glutaminylcysteine.

N-Benzylcarbonylcystine benzyl ester. *N*-Benzylcarbonylcystyl chloride from 20 g. of *N*-benzylcarbonylcystine [Harington & Mead, 1935] was made into a thick paste with ether and added to a mixture of benzyl alcohol (26 g.: 200 % excess) with 2 vol. of dry chloroform. Next day the filtered solution was evaporated under diminished pressure and the syrupy residue dissolved in

ethyl acetate. The ethyl acetate solution was washed with water, saturated NaCl, sodium bicarbonate solution and finally twice with saturated NaCl. It was then dried with anhydrous sodium sulphate, concentrated to a syrup and stirred well with 5-10 vol. of ligroin. After standing for a short time in the refrigerator the lower layer had set to a firm jelly, from which the ligroin was decanted. The lower layer was ground with ligroin and collected. The substance was dried *in vacuo* over several changes of sulphuric acid. Yield 22.7 g. = 84 % of a rather sticky amorphous powder.

Cysteine benzyl ester hydroiodide. *N*-Benzylcarbonylcysteine benzyl ester (13.9 g.) in glacial acetic acid (140 ml.) was treated with phosphonium iodide (22.8 g.) at 50° in the manner described by Harington & Mead [1935]. After 70 min. evolution of carbon dioxide was almost at an end. The solution was then decanted from excess reducing agent and concentrated under diminished pressure to a small volume. Crystallization of the product was started by scratching and was completed by the cautious addition of dry ether, and by keeping at 0° for 2 hours. The product was collected and washed with acetic acid and dry ether. It was recrystallized from glacial acetic acid and formed colourless plates melting with decomposition at 139° after sintering at 135°. (Found: N, 4.4 %; I, 36.6, 37.4 %. $C_{10}H_{14}O_2NIS$ requires N, 4.1; I, 37.5 %).

N-Benzylcarbonyl- α -glutamylcysteine benzyl ester. Cysteine benzyl ester hydroiodide (10.15 g. of recrystallized material) was suspended in dry chloroform (40 ml.). The suspension was cooled in ice and treated with diethylamine (3.0 ml.) which caused the hydroiodide to pass into solution. To the latter cooled in ice-salt was added a solution of *N*-benzylcarbonylglutamic anhydride (7.9 g.) in dry chloroform (30 ml.). The solution was kept in the freezing mixture for 30 min. and then overnight at room temperature. The partly gelatinous material which had separated was collected and the filtrate evaporated to dryness under diminished pressure. The residue together with the first crop was crystallized from boiling propyl alcohol and recrystallized from diluted ethyl alcohol. The product formed colourless elongated prisms melting at 148°. Yield 9 g. = 64 % of the theoretical. (Found: N, 5.85; S, 6.88 %; $C_{23}H_{26}O_7N_2S$ requires N, 5.9; S, 6.75 %.)

N-Benzylcarbonyl- α -glutamyl-S-benzylcysteine benzyl ester. *N*-Benzylcarbonyl- α -glutamylcysteine benzyl ester (3.0 g. of recrystallized material) was suspended in dry chloroform (7.5 ml.) and dissolved by addition of diethylamine (1.3 ml.: 2 mol.). The solution was treated with benzyl bromide (0.92 ml.: 1 mol.). The temperature rose to about 40° after the addition. After about 10 min. further quantities of diethylamine (0.26 ml.) and benzyl bromide (0.2 ml.) were added. When the mixture had stood at room temperature for a further 45 min. it was evaporated to a syrup under diminished pressure. The syrup was dissolved in alcohol and the evaporation repeated in order to remove the last traces of chloroform. The residue was dissolved in a small volume of alcohol and the solution was treated with a slight excess of HCl (2*N*) and then cautiously with water. After a short time at 0° the crystalline product was collected and washed with alcohol. It was recrystallized from *n*-propyl alcohol with the use of norite. Yield 3.0 g. or 79 % of the theoretical. The substance forms colourless needles or prisms melting at 144°. (Found: N, 4.9; S, 5.41 %. $C_{30}H_{32}O_7N_2S$ requires N, 4.96; S, 5.6 %. 45.7 mg. required 4.06 ml. *N*/50 NaOH to neutralize to cresol red; $C_{30}H_{32}O_7N_2S$ titrating as a monobasic acid requires 4.05 ml.)

N-Benzylcarbonyl- α -glutamyl-S-benzylcysteine benzyl ester. *N*-Benzylcarbonyl- α -glutamyl-S-benzylcysteine benzyl ester (2.3 g. of recrystallized material) was suspended in dry ether (25 ml.). Phosphorus pentachloride (1.2 g.; 15 %

excess) was added to the suspension which was cooled intermittently in ice-salt and vigorously shaken. Separation of the acid chloride as fine needles started before the suspension had become quite clear. Shaking was continued for about 10 min. and light petroleum (50 ml. of B.P. 60–80°) was then added in order to complete the separation of the product. The latter was collected, washed with light petroleum, and suspended in dry chloroform (40 ml.). The suspension was added slowly with constant agitation to ether (100 ml.) at –10° which had been previously saturated with dry ammonia at that temperature. A white granular precipitate settled out. Any lumps were broken up with a rod and the mixture was left at room temperature overnight. The precipitate was collected, washed with ether and dried at 40°. It was then ground with water, collected, washed with ether and dried. The product was recrystallized from *n*-propyl alcohol (150 ml.) with the use of norite. Yield 1.6 g. or 70% of the theoretical. The substance forms fine needles melting at 182° after sintering. (Found: N, 7.47; S, 5.74%. $C_{30}H_{33}O_6N_3S$ requires N, 7.46; S, 5.68%.)

Glutaminylcysteine. Removal of the *N*-benzylcarbonyl-, *S*-benzyl- and benzyl ester-groups from the preceding compound was accomplished by reduction with sodium in liquid ammonia [Siffert & du Vigneaud, 1935]. No tendency was observed for the benzyl ester group to be replaced under these conditions by an amide group.

N-Benzylcarbonyl- α -glutaminyl-*S*-benzylcysteine benzyl ester (5.0 g.) was suspended in liquid ammonia (*ca.* 50 ml.) in an apparatus fitted with a mechanical stirrer. The suspension was treated with sodium in small pieces (1.23 g. : 6 equiv.). The sodium was added rapidly at first and the mixture kept at –60° during this period. The last portions of sodium were added more slowly while the temperature was allowed to rise to the boiling point of ammonia. A deep blue colour due to excess of sodium appeared soon after the addition of the last piece. The crude product was isolated exactly as in the case of cysteylglutamine, the total yield being 60%.

The crude peptide was suspended in a small volume of water and treated with 2*N* H_2SO_4 until solution was almost complete. After filtration the solution was treated with an excess of Denigès mercuric sulphate reagent. The mercury compound was collected at the centrifuge and a second crop obtained by dilution of the mother liquor with water. The precipitates were united, washed with water several times, suspended in water and gassed with H_2S during several hours. The mercuric sulphide was centrifuged, resuspended in water, again treated with H_2S , and finally centrifuged. The combined aqueous liquors were evaporated to a small volume under diminished pressure, then warmed to about 35° and treated with a suspension of cuprous oxide. The latter dissolved but no precipitate appeared until several additions of the reagent had been made and the inside of the tube had been scratched with a rod. The faintly yellow and rather soluble cuprous derivative was centrifuged and washed. It was found possible to avoid undue loss by interposing an aqueous alcoholic washing after every second washing with water. The water-alcohol treatment consisted in suspending the material in a small volume of water and then adding about 2 vol. of alcohol. After more than 12 washings the liquor remained cloudy after centrifuging although it still gave a precipitate with HCl and $BaCl_2$. The cuprous compound was suspended at this stage in water and decomposed with H_2S . The cuprous sulphide was centrifuged and washed with water. The combined solution and washings were concentrated to a small volume under diminished pressure and transferred to a small basin. Crystallization set in as soon as the basin was scratched. The pH was adjusted to 4.5 by the addition of 5*N* ammonia and concentration continued over phosphoric

oxide *in vacuo*. After a night at 0° the peptide was collected, washed with water and dried at 56° *in vacuo*. Yield 264 mg. By concentrating the mother liquor a quantity of less pure material was obtained.

The peptide formed colourless rectangular plates or prisms which decomposed sharply at 197°. (Found: C, 38·7; H, 6·20; N, 16·0, 16·1; S, 12·85; amide N, 5·28 %. $C_8H_{15}O_4N_3S$ requires C, 38·6; H, 6·02; N, 16·9; S, 12·85; amide N, 5·6 %.)

It is evident from the analytical data that this peptide was not quite pure. An attempt was made to purify it by recrystallization from water followed by aqueous alcohol. The product however proved on analysis to be less pure than the starting material. This result is less surprising when account is taken of the great lability of the amide nitrogen in this compound (Table II).

Glutaminy compounds exhibit a tendency to lose ammonia and undergo cyclization to derivatives of pyrrolidone [Bergmann & Zervas, 1933]. Evidence that no such change had occurred during the preparation of glutaminylcysteine is provided by the results of the amide nitrogen determination. Free pyrrolidone-carboxylcysteine would itself give no amide nitrogen but would form an ammonium salt which might bear a superficial resemblance to glutaminylcysteine. The ammonium salt would however liberate ammonia when dissolved in an alkaline buffer solution and distilled *in vacuo* under the conditions for the determination of amide nitrogen but without previous heating in acid solution. Two experiments made to test this point showed that only a negligible amount of ammonia was liberated by the peptide under these conditions.

Optical rotation. Glutaminylcysteine. 15·3 mg. in 0·5 ml. of water had $\alpha_{5461} - 0·15^\circ$ in a 0·5 dm. tube whence $[\alpha]_{5461} = -9·8^\circ$.

Glutaminylcysteine. On account of the insolubility of this substance the determination was made in acid solution. To a solution of glutaminylcysteine (12·6 mg.) in water containing the theoretical quantity of hydrogen peroxide (1·11 ml.) was added HCl (0·25 ml. of 0·25 *N*), containing a trace of copper sulphate. When oxidation was complete the solution had $\alpha_{5461} - 0·55^\circ$ in a 0·5 dm. tube giving $[\alpha]_{5461} = -119^\circ$.

Iodine titration. The peptide (9·8 mg.) was dissolved in 5 % potassium iodide solution (5 ml.) and treated with approximately 0·01 *N* iodine solution (5·0 ml.). The excess of iodine was back-titrated with 0·01 *N* sodium thiosulphate. 4·68 ml. of 0·01 *N* iodine were required as against the theoretical amount of 3·94 ml. The high result obtained in this titration may be compared with that obtained by Harington & Mead [1935] for glutamylcysteine in which the cysteine is linked in an analogous manner.

Determination of labile sulphur.

The apparatus was similar to that described by Abel & Geiling [1925]. Preliminary experiments showed that a rubber stopper, if purified by sodium hydroxide solution in the usual way, liberated no sulphur when new. After being in use for some time, however, it became responsible for a large blank. The apparatus finally employed was therefore made entirely of glass.

Control of method. A freshly prepared solution of H_2S in ice-cold, oxygen-free NaOH was standardized by the procedure described by Treadwell & Hall [1919]. Portions (2·0 ml.) of this solution were boiled with water (3 ml.) and 0·2 *N* Na_2CO_3 (5 ml.) in the 30 × 150 mm. pyrex tube of the apparatus. A slow stream of nitrogen passed through the solution and reflux condenser and escaped through ammoniacal cadmium chloride solution contained in a 100 ml. conical flask. When 45 min. had elapsed, 5 ml. of approximately 5 *N* HCl were added to the sulphide solution through a tap funnel and ebullition was continued in a

more rapid stream of nitrogen for 30 min. The receiving flask was then disconnected, the contents were diluted with water (25 ml.) and the cadmium sulphide was dissolved by the addition of 5 *N* HCl which was immediately followed by an excess of 0.01 *N* alkaline potassium iodate solution containing potassium iodide. The excess iodine was ultimately titrated with 0.01 *N* sodium thiosulphate solution.

The standard sulphide solution contained 0.111 mg. S per ml. by direct titration. The results from three control experiments were 0.111, 0.113 and 0.111 mg./ml.

Determination of labile sulphur in peptides. The peptide (about 16 mg.) was weighed into a standard 10 ml. flask, dissolved in a small volume of water, and treated with the theoretical quantity of hydrogen peroxide in the presence of a trace of ferric ammonium sulphate. When oxidation was complete the solution was made up to 10 ml. Portions (2.0 ml.) of this solution were treated as described above.

Determination of labile sulphur in insulin. A solution of crystalline insulin was standardized by the determination of nitrogen on aliquot portions (Kjeldahl). The labile sulphur in 2 ml. of this solution was determined as described above with the exception that capryl alcohol was added in order to diminish the frothing. A blank experiment showed that the result was thereby unaffected.

Determination of the amount of ammonia liberated by glutaminylcystine and cystylglutamine during treatment with acid or alkali.

Standard solutions, containing approximately 1.5 mg. per ml. of glutaminylcystine or cystylglutamine were prepared freshly for each experiment. Three 2 ml. aliquots of peptide solution were measured into test-tubes and treated with the theoretical quantity of hydrogen peroxide and a trace of copper sulphate. Two portions were used for duplicate determinations and the third as a control for the oxidation.

Acid treatment. The oxidized peptide solutions were mixed with an equal volume of 0.2 *N* HCl and were heated in a boiling water bath for 3 hours. During this time the tubes were closed by stoppers provided with short lengths of capillary tube. Before analysis the solutions were almost neutralized by the addition of 0.4 *N* NaOH.

Alkaline treatment. The oxidized peptide solutions were mixed with an equal volume of *N*/15 NaOH. Each tube was closed by a stopper which carried a small drying tube containing glass beads moistened with *N* HCl (0.2 ml.). After 3 hours in a thermostat at 34° the beads were rinsed with water. The rinsings were added to the contents of the tube and the now slightly acid solutions were washed into the ammonia distillation apparatus. Control experiments with a standard solution of ammonium sulphate showed that no loss of ammonia was incurred by this procedure.

Distillation of ammonia. The apparatus resembled that described by Parnas & Heller [1924]. The steam supply was regulated automatically by means of a piece of capillary tubing in the steam inlet. The vacuum was similarly controlled by a capillary leak inserted into the connection between the pump and the vessel enclosing the receiver. A side-arm on this vessel permitted the tip of the condenser to be rinsed into the receiver. The water-cooled condenser was of glass and gave rise to a constant blank equivalent to 0.007 mg. N. Truncated monax test-tubes (18 × 90 mm.) served as receivers.

For a distillation a receiver containing 0.01 *N* HCl (2.00 ml.) was adjusted so that the tip of the condenser ended just beneath the surface of the acid. Phos-

phate-borate buffer (5 ml. of a solution containing 27.2 g. KH_2PO_4 and 9.55 g. $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$ in a litre) was added to the sample in the distillation flask and the latter was connected to the apparatus by a greased joint. Sodium hydroxide-borax mixture (3 ml. of a solution containing 50 g. $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$ in a litre of 0.5 *N* NaOH) was added through a side-arm on the steam supply tube and the apparatus was evacuated. Distillation was maintained for 3 min. with the end of the condenser dipping beneath the surface of the liquid in the receiver and for 1 min. with the receiver lowered. In all 5 ml. of distillate were collected. Steam was then shut off and air admitted. The tip of the condenser was rinsed and the contents of the receiver were treated with potassium iodide solution (2 ml. of 5 %) and potassium iodate solution (3 drops of 4 %). After 3 min. the iodine was titrated with 0.01 *N* sodium thiosulphate.

Blank estimations on all the reagents and also an analysis of a standard ammonium sulphate solution were made before every determination of ammonia.

Determinations of amide nitrogen.

Ovalbumin. The following determinations of the amide nitrogen content of ovalbumin were made in order to check the accuracy of the method. The ovalbumin, which had been recrystallized by the use of sodium sulphate, was kindly provided by Dr Neuberger.

Method of hydrolysis	Equivalents of amide N per molecule
20 % HCl at 100° 3 hours	26.1
0.2 <i>N</i> HCl at 100° 20 hours	24.6, 24.4

The most recently determined value is 24 equivalents per molecule [Shore *et al.*, 1935].

Insulin. The crystalline insulin, which was kindly provided by Dr D. A. Scott, contained 9.2 % of moisture and 0.85 % ash. Approximately 15 mg. portions were weighed into test-tubes and were dissolved in 5 *N* HCl (4 ml.). It was found to be more advantageous to add water first (0.73 ml.) and then HCl of constant B.P. (3.27 ml.). The insulin was allowed to dissolve completely after the addition of the first drop of acid and the remainder was then run in.

The tubes, which were closed by stoppers carrying micro-reflux condensers, were heated for varying periods in a boiling water-bath. For analysis, each solution was washed into the distillation vessel and treated with phosphate-borate buffer (5 ml.) and with 10 *N* NaOH (1.85 ml.). 5 drops of bromothymol blue were added followed by sufficient 0.4 *N* NaOH to give a greenish yellow tint similar to that given by the buffer and indicator alone. The flask was then attached to the apparatus, sodium hydroxide-borax mixture (3 ml.) was added and the ammonia distilled in the usual manner.

APPENDIX.

The preparation and properties of the following substances which may prove to be of use in peptide synthesis are described below.

Glycine benzyl ester hydrochloride.

N-Benzylcarbonylcystylglycine benzyl ester.

α -Benzyl ester of *N*-benzylcarbonyl- γ -glutamylglycine benzyl ester.

N-Benzylcarbonyl- β -aspartylglycine.

α -Benzyl-*N*-benzylcarbonyl- β -aspartylcystine ethyl ester.

γ -Methyl-*N*-benzylcarbonyl- α -glutamyl-*S*-methylcysteine benzyl ester.

Glycine benzyl ester hydrochloride. Glycyl chloride hydrochloride prepared from 25 g. of glycine by the method of Fischer [1905] was added slowly to an ice-cold mixture of benzyl alcohol (40 ml.) and ether (40 ml.). More ether was added while the product was crystallizing from the mixture.

After a night at 0° the substance was collected and washed with ether. The yield was 48 % of the theoretical.

For purification the crude material (15 g.) was dissolved in water (35 ml.). The solution was washed with ether in order to remove a trace of benzyl chloride, cooled in ice-salt treated with anhydrous potassium carbonate (20 g.) and extracted with ether at the centrifuge. The aqueous layer was made into a sludge with more potassium carbonate and extracted twice more with ether. The ethereal extracts were united, dried over anhydrous potassium carbonate in the presence of norite, and treated with dry gaseous HCl until separation of the product was complete. The substance was collected and washed with ether. Glycine benzyl ester hydrochloride forms long colourless prisms melting at 139–140°. (Found: N, 6.94; Cl, 17.6 %. $C_9H_{12}O_2$ NCl requires: N, 6.96; Cl, 17.6 %.)

N-Benzylcarbonylcystylglycine benzyl ester. Glycine benzyl ester hydrochloride (9.6 g.) in a small volume of water was cooled in ice-salt and treated with anhydrous potassium carbonate (10 g.). The free ester was extracted with three portions of ethyl acetate. The ethyl acetate solution was dried over sodium sulphate, cooled in ice-salt and treated with *N*-benzylcarbonylcystyl chloride from 20 g. of *N*-benzylcarbonylcystine. After standing for several hours at room temperature the product was collected and washed with ethyl acetate and with ether and dilute acid. It was recrystallized from *n*-propyl alcohol and formed large prismatic needles melting at 167–168°. (Found: N, 6.9; S, 8.03 %. $C_{40}H_{42}O_{10}N_4S_2$ requires: N, 6.98; S, 7.98 %.)

α-Benzyl ester of N-benzylcarbonyl-γ-glutamylglycine benzyl ester. A solution of glycine benzyl ester in ethyl acetate was prepared as described above from glycine benzyl ester hydrochloride (6.5 g.). The solution was cooled in ice-salt and was treated with the acid chloride obtained by the method of Bergmann *et al.* [1933] from *α*-benzyl-*N*-benzylcarbonylglutamate (5 g.). (This acid chloride has been obtained crystalline in the form of small needles.) After standing for several hours the solution was filtered from precipitated glycine benzyl ester hydrochloride, washed with dilute acid and with water, dried over sodium sulphate and concentrated to a small volume under diminished pressure. The product which was precipitated by the cautious addition of ligroin was recrystallized once from ethyl acetate-ligroin and then twice from ethyl alcohol. It formed cigar-shaped crystals melting at 104–105°. (Found: C, 66.8; H, 5.90; N, 5.45 %. $C_{23}H_{30}O_7N_2$ requires: C, 67.2; H, 5.79; N, 5.41 %.)

N-Benzylcarbonyl-β-aspartylglycine. The acid chloride prepared by the method of Bergmann *et al.* [1933] from *N*-benzylcarbonylaspartic acid *α*-benzyl ester (3.0 g.) was dissolved in dry chloroform (15 ml.). This solution and *N* NaOH (8.5 ml.) were added in parallel portions during 60 min. to a well stirred solution of glycine (0.63 g.) in *N* NaOH (8.5 ml.). Stirring was continued for 15 min. after the last addition and then the mixture was centrifuged. The aqueous layer was washed with ether, made acid to Congo red, saturated with ammonium sulphate and extracted thoroughly with ethyl acetate. The ethyl acetate solution was dried over sodium sulphate, concentrated to a small volume under diminished pressure and treated with ligroin. The oil which separated soon hardened. The product was collected and crystallized from acetic acid-benzene. *N*-Benzylcarbonyl-β-aspartylglycine forms fine needles melting with decomposition at 153° after sintering. (Found: C, 51.6; H, 5.17; N, 8.4 %. $C_{14}H_{16}O_7N_2$ requires: C, 51.9; H, 4.94; N, 8.6 %.)

*α-Benzyl-*N*-benzylcarbonyl-β-aspartylcystine ethyl ester.* The acid chloride prepared from *N*-benzylcarbonylaspartic acid *α*-benzyl ester (3 g.) was shaken with a suspension of cystine ethyl ester hydrochloride (1.56 g.) in dry pyridine (10 ml.) and cooled in ice-salt. After 4 hours at room temperature 2 vol. of chloroform were added and the solution was filtered from excess cystine ethyl ester hydrochloride. The filtrate was evaporated to a syrup under reduced pressure and was treated with successive portions of water until it solidified. The material was dissolved in hot ethyl acetate, precipitated by ligroin and then crystallized from *n*-propyl alcohol. It formed colourless needles melting at 160°. (Found: N, 5.7; S, 6.56 %. $C_{48}H_{54}O_{14}N_4S_2$ requires: N, 5.75, S, 6.57 %.)

*γ-Methyl-*N*-benzylcarbonyl-α-glutamylcystine benzyl ester.* An ethereal suspension of *N*-benzylcarbonyl-α-glutamylcystine benzyl ester was treated with an excess of diazomethane at 0°. The acid passed into solution with evolution of nitrogen. After 60 min. the solution was filtered from a small precipitate and evaporated to dryness. The residue crystallized from ethyl acetate-ligroin. m.p. 85–86°. (Found: C, 59.6; H, 5.92; N, 5.8 %. $C_{25}H_{30}O_7N_2S$ requires C, 59.8; H, 5.98; N, 5.6 %.)

SUMMARY.

The syntheses of glutamylcysteine and cysteylglutamine have been described.

In view of the suggestion of Jensen & Evans [1934] these peptides have been tested for hypoglycaemic activity and compared in their chemical behaviour with insulin.

Neither peptide in reduced or oxidized form exhibited any hypoglycaemic activity. The peptides showed a general resemblance to insulin in respect of the lability of their amide nitrogen but differed from insulin in that their sulphur was much more stable towards alkali.

The time course of liberation of ammonia from insulin by 5*N* hydrochloric acid at 100° indicates the presence of about 34 amide groups per mol. From this value together with published titration data it is concluded that insulin contains about 30 % of glutamic acid.

The preparation and properties of some intermediates for peptide synthesis are recorded.

REFERENCES.

- Abel & Geiling (1925). *J. Pharmacol.* **25**, 423.
—— ——— Rouiller, Bell & Wintersteiner (1927). *J. Pharmacol.* **31**, 65.
Bergmann & Zervas (1932). *Ber. dtsh. chem. Ges.* **65**, 1192.
—— ——— (1933). *Hoppe-Seyl. Z.* **221**, 51.
—— ——— & Salzmann (1933). *Ber. dtsh. chem. Ges.* **66**, 1288.
Brand & Sandberg (1926). *J. biol. Chem.* **70**, 381.
Fischer (1905). *Ber. dtsh. chem. Ges.* **38**, 2914.
Harington & Scott (1929). *Biochem. J.* **23**, 384.
—— & Mead (1935). *Biochem. J.* **29**, 1602.
—— & Neuberger (1936). *Biochem. J.* **30**, 809.
Jensen & Evans (1932). *Hoppe-Seyl. Z.* **209**, 134.
—— ——— (1934). *Physiol. Rev.* **14**, 188.
—— & Wintersteiner (1932). *J. biol. Chem.* **97**, 93.
—— Evans, Pennington & Schock (1936). *J. biol. Chem.* **114**, 199.
Parnas & Heller (1924). *Biochem. Z.* **152**, 1.
Shore, Wilson & Stueck (1935). *J. biol. Chem.* **112**, 407.
Sifferd & du Vigneaud (1935). *J. biol. Chem.* **108**, 753.
Treadwell & Hall (1919). *Analytical Chemistry*, 5th ed., Vol. 2, p. 637.
(John Wiley & Sons, New York.)
du Vigneaud, Audrieth & Loring (1930). *J. Amer. chem. Soc.* **52**, 4500.
Wintersteiner, du Vigneaud & Jensen (1928). *J. Pharmacol.* **32**, 397.