XXV. THE AMINO-ACIDS OF GLUTENIN.

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The necessity for investigating new methods of protein analysis requires no emphasis. While the separation of the bases by the method of Kossel and Patten [1903], especially as recently modified by Vickery and Leavenworth [1928], and the precipitation of the dicarboxylic acids by calcium hydroxide and alcohol [Foreman, 1914] lead to nearly quantitative results, the isolation of the monoamino- and non-amino-monocarboyxlic acids by the usual ester method leaves much to be desired both from the point of view of purity of products and of yield. The different modifications of the ester method that have been devised have been successful in overcoming certain of its shortcomings. Preliminary extraction of the amino-acids as recommended by Dakin [1918] and the use of their lead salts for esterification adopted by Foreman [1919] lead to anhydrous preparations of the monoamino-acids which are therefore more readily esterified.

However in any separation of the amino-acids based on the fractionation of their esters certain serious drawbacks inherent in the method are bound to remain, *viz.* (i) loss of esters due to volatilisation and to decomposition during distillation, (ii) the difficulty of separating closely related substances which may appear in the same fraction of the distillate, and (iii) the presence of unmanageable syrups which hinder purification and crystallisation. In this connection Osborne's observations [1910] on the separation of a synthetic mixture of pure amino-acids—in which the recovery was only 72·3 %—are of significance. It is clear then that a successful method for the separation of amino-acids which does not involve the preparation and distillation of the esters would form a valuable contribution to the study of the amino-acid composition of the proteins.

Some of the results of a series of investigations carried out in this laboratory under the direction of the late Professor Schryver with the object of separating the amino-acids by taking advantage of the differences in the solubilities of their copper salts have already been published by Town [1928] and by Brazier [1930]. In the present paper the results of experiments on the separation of the copper salts of the amino-acids from wheat glutenin are described.

It has been possible to obtain higher yields of alanine, valine, proline and aspartic acid than those previously obtained by the ester method. Since the

completion of this work, the publication of which has for various reasons been considerably delayed, two of the increased values have been confirmed in other laboratories. Spörer and Kapfhammer [1930], using Reinecke salt for the precipitation of proline, have obtained a value (5·98 %) practically identical with that found in this investigation (6·15 %). Jones and Moeller [1928], who have used essentially the same procedure as that described in this paper for the isolation of the dicarboxylic acids, have found 2 % aspartic acid in close agreement with the value (1·85 %) now reported. Phenylalanine and leucine are found in the same fraction in both the copper salt and the ester method, and they have not been satisfactorily separated. The actual yields of these two substances are lower than those found by Osborne, but the use of an indirect method involving the oxidation of phenylalanine to benzoic acid has raised the value of phenylalanine to 2·75 % (ester method, 1·97 %).

Apart from the higher yields of certain individual amino-acids it has been possible to trace a large proportion of the nitrogen not accounted for in the products isolated. The nitrogen entirely unaccounted for amounts to only about 8 % of the total, a loss which can hardly be avoided considering the nature of the processes involved. By checking the amount of nitrogen lost by adsorption on various precipitates it has been found possible to gain some idea of the deficiencies that fall upon particular amino-acids. By this means also it has been possible to devise modifications of the method so as to obviate such losses in future hydrolyses.

Separation of the amino-acids is effected by taking advantage of the difference in solubility of their copper salts in water and in methyl alcohol. The extraction, under carefully regulated conditions, of the dry copper salts of the mixed amino-acids by these two solvents in succession gives three fractions.

(i) Copper salts soluble in methyl alcohol: *valine* and *proline*.

(ii) Copper salts insoluble in methyl alcohol but soluble in water under the conditions of the experiment: *glycine*, *alanine*, *aspartic acid*, *glutamic acid*, *arginine*, *histidine*, *lysine* and some *tyrosine*.

(iii) Copper salts insoluble in methyl alcohol and in water: *leucine*, *phenylalanine* and *aspartic acid*.

In each of the above fractions, especially (i) and (ii), a certain amount of unidentified matter was also present. The amino-acids in each fraction have been separated by taking advantage of the difference in solubility either of the acids themselves or of their derivatives in suitable solvents.

Experimental.

Preparation of the glutenin. Gluten, obtained by thorough washing of wheat flour, was repeatedly extracted with 73 % alcohol to remove the gliadin. The residue was dried at room temperature, ground to a fine powder and exhaustively extracted with alcohol. Excess of alcohol was removed at room

temperature with the help of a fan and the residue was dissolved in 0·2 % sodium hydroxide. The solution was filtered clear and precipitated by the addition of dilute acetic acid. The product was purified by reprecipitation from sodium hydroxide. It was then washed free from sodium acetate with dilute alcohol and finally dehydrated with acetone. The glutenin thus prepared contained 8·36 % water and 0·44 % ash, and, ash- and moisture-free, 17·02 % nitrogen.

Hydrolysis. 250 g. of glutenin equivalent to 228 g. of ash- and moisture-free protein and to 38·81 g. of nitrogen were hydrolysed by boiling with four times the weight of 25 % sulphuric acid under a reflux condenser for 24 hours. The cooled solution was diluted to 4 litres and the humin was filtered off, washed thoroughly with hot water and analysed for nitrogen. Ammonia was determined in a small aliquot by aeration. Most of the sulphuric acid was now precipitated by the addition of saturated baryta to the hydrolysate. The barium sulphate was repeatedly washed with hot water, then dried at 110° and washed again after powdering. A nitrogen determination on the barium sulphate showed that it retained 6·47 % of the total nitrogen of the protein. The filtrate and washings from the barium sulphate were concentrated to about 2 litres and the sulphuric acid was quantitatively removed. On further concentration to 750 cc. a precipitate of crude tyrosine separated which gave, after recrystallisation, 6·84 g. of the pure amino-acid.

Preparation of the dry copper salts. The filtrate from the tyrosine was heated on a water-bath in a large porcelain dish and treated with copper carbonate in small portions at a time. After the addition of a fairly large excess of copper carbonate the solution was evaporated to a thick syrup which was left on the water-bath for some hours. It was then taken up again in water and filtered from the excess of copper carbonate, and the latter was washed thoroughly with boiling water. The filtrate and washings were once more evaporated to a syrup with excess of copper carbonate to ensure complete conversion into the copper salts. After again taking up in water and filtering, the solution was evaporated to a thick syrup, allowed to cool and then stirred up with a liberal amount of acetone. After standing for a short time the acetone was poured off and replaced by a fresh quantity. On repeating this process three to four times a dark blue friable mass was obtained which was easily ground to a fine powder. This was filtered with suction and freed from acetone in a vacuum desiccator over sulphuric acid. It was then dried thoroughly at 110° for 24 hours. If the syrup is evaporated too far before granulation with acetone or if an appreciable amount of acetone is present in the mixture when placed in the drying oven hard cakes are obtained which are difficult to break up and impossible to dehydrate completely.

The thoroughly dried copper salts weighed 279 g. and contained 26·53 g. of nitrogen.

Fractionation of the copper salts.

(*a*) *Extraction with methyl alcohol.* The thoroughly dried and finely ground copper salts were shaken with three times their volume of dry methyl alcohol (freshly distilled from sodium) on a shaking machine. After about 2 hours the blue solution was filtered and fresh methyl alcohol was added to the residue. Seven extractions were usually sufficient to remove all soluble material. The combined extracts were filtered at the pump, evaporated to a syrup and the residue was granulated with acetone. After drying for one hour at 100° and taking up once more in methyl alcohol a small residue was usually obtained which was put back into the main fraction of copper salts. The solution when evaporated gave a residue completely soluble in methyl alcohol.

(*b*) *Extraction with water.* The residual copper salts were extracted four times successively with large volumes of water at 60°. The aqueous solutions were evaporated and the residue was dehydrated with acetone. The extraction was repeated on this residue, using very large volumes of warm water. Any insoluble residue was added to Fraction III consisting of the copper salts insoluble in both methyl alcohol and water. The three fractions were decomposed with hydrogen sulphide, I and II in aqueous solution, III in dilute sulphuric acid. The copper sulphide obtained in each case was very thoroughly washed and after drying was analysed for nitrogen. The filtrate and washings in each fraction were evaporated *in vacuo* to remove hydrogen sulphide and the nitrogen was determined in an aliquot in each case.

Fraction I. Amino-acids from copper salts soluble in methyl alcohol.

On evaporating the aqueous solution a pale brown sticky mass was obtained. This was thoroughly dehydrated by repeated addition of absolute alcohol and evaporation *in vacuo*. It was next exhaustively extracted with hot absolute alcohol. The insoluble residue was white and friable. The alcoholic extracts were evaporated and the residue was re-extracted with alcohol; a small amount of undissolved material was added to the main insoluble portion. This operation was repeated until the material obtained on evaporation was completely soluble in absolute alcohol.

Valine. The residues insoluble in ethyl alcohol were combined and reconverted into their copper salts. The material recovered by decomposition of the latter was entirely insoluble in alcohol. When recrystallised from water it had M.P. 313–315°, N 11·92 %, and was therefore valine. Yield 2·33 g. No alcohol-soluble zinc salt suggestive of hydroxyvaline [Schryver and Buston, 1926] could be obtained.

Proline. The material completely soluble in alcohol was recovered by evaporation and taken up in water. The solution was analysed for non-amino-nitrogen, diluted until the concentration of the latter was about 1 g. per 100 cc., brought to the boil and treated with a hot solution containing sufficient picric acid to combine with the non-amino-nitrogen present [Town,

1928]. After evaporating and cooling proline picrate crystallised out in the form of golden yellow needles. On further evaporation of the mother-liquor a second small crop was obtained. The proline picrate was washed at the pump with cold water and, after drying in the desiccator, was extracted with ether to remove excess of picric acid as well as another picrate found to be soluble in ether (see later).

The aqueous and ethereal filtrates from the proline picrate were freed from picric acid by means of sulphuric acid and ether. After the quantitative removal of sulphuric acid the solution was evaporated to a syrup, taken up in 95 % alcohol and treated with a saturated solution of cadmium chloride [Kapfhammer and Eck, 1927] which was cautiously added drop by drop until no further precipitation took place. The precipitate was filtered off and washed with cold 95 % alcohol until free from chloride, re-dissolved in water and freed from cadmium chloride (chloride precipitated by silver sulphate, and silver and cadmium by hydrogen sulphide). After the quantitative removal of sulphuric acid the solution was once more treated with picric acid. The combined proline picrate precipitates were recrystallised from water, when 42 g. of picrate melting at 148° were obtained corresponding to 14·02 g. of proline.

The mother-liquors from which proline had been recrystallised as the picrate and the residual proline had been precipitated as the cadmium chloride compound were freed quantitatively from all reagents and the aqueous solution was evaporated to a syrup. It contained 5·91 % of the total nitrogen. No crystallisation took place on long standing in the desiccator, and it was found impossible to obtain it in a dry state. No definite compound was isolated from this mixture, but it could be separated into two fractions, one forming a picrate soluble in ether, the other not giving a picrate.

Fraction II. Amino-acids from copper salts insoluble in methyl alcohol, but soluble in water.

Tyrosine. On evaporation of the amino-acid solution to about 600 cc. a small amount of tyrosine crystallised out. The weight of pure recrystallised material was 2·76 g., making with that previously isolated 9·6 g. of tyrosine in all.

Aspartic and glutamic acids. The solution was treated according to Kingston and Schryver's modification [1924] of Foreman's [1914] method for the isolation of the dicarboxylic acids. It was found that by adding baryta in a thin cream in fair excess and keeping the mixture vigorously stirred during the slow and gradual addition of alcohol the formation of a sticky precipitate could be avoided. The barium salts were re-dissolved in water and again precipitated with alcohol in the presence of excess of baryta. The filtrate and washings from this second precipitation were not added to the main filtrate but were examined separately. The precipitate was dissolved in water and the barium was removed quantitatively. The crystals obtained from this

solution on evaporation, though possessing the characteristic form of glutamic acid, were found to be impure and therefore they were fractionated by the procedure adoped by Dakin [1919]. Glutamic acid was separated in three successive crops as the hydrochloride, 72·65 g. of recrystallised hydrochloride, corresponding to 58·14 g. of glutamic acid, being obtained.

From the mother-liquors after removal of glutamic acid aspartic acid was separated as the lead salt [Dakin, 1919]. The yield of pure recrystallised aspartic acid was 0·52 g.

By treatment of the filtrate from the lead aspartate with sodium hydroxide and silver nitrate the silver salt of hydroxyglutamic acid was precipitated. It was not however found possible to obtain crystals of hydroxyglutamic acid even after long standing in the vacuum desiccator. The hard, dry mass thus obtained weighed 2·3 g. corresponding to 1 % of the weight of glutenin. It contained 0·54 % of ash. (N: found, 8·40 %; calc., 8·59 %.)

Glycine. The filtrate and washings obtained from the second precipitation of the barium dicarboxylates were freed from alcohol and barium. The solution gave a negative reaction with Millon's reagent showing the absence of tyrosine. A small precipitate was obtained with phosphotungstic acid. It was filtered off, decomposed and the solution so obtained was added to the main filtrate from the dicarboyxlic acid precipitation. The filtrate was freed from phosphotungstic acid and the solution was evaporated to dryness. The residue obtained consisted of glycine mixed with a certain amount of inorganic impurity. By recrystallisation 0·6 g. of pure glycine was obtained. (N: found, 18·57 %; calc., 18·67 %.) It was further identified by conversion into the picrate M.P. 189°.

Removal of the bases. In the main filtrate from the dicarboxylic acid precipitate arginine and histidine were precipitated as their silver salts by the method of Vickery and Leavenworth [1928]. The nitrogen in the solution obtained by decomposing the silver precipitate was 11·6 % of the total in good agreement with Osborne's [1910] value of 11·39 %. From the solution, after removal of arginine and histidine, lysine was separated in the usual way as picrate. After its removal the residual solution still contained 1 g. of nitrogen, which was not identified.

Alanine and glycine. The solution with the washings from the phosphotungstic acid precipitation above was freed from phosphotungstic and sulphuric acids and evaporated to 500 cc., and the amino-acids were converted into their barium carbamates by the method of Kingston and Schryver [1924]. The precipitate was dissolved in water at room temperature and freed from barium. On evaporation glycine (1·13 g.) crystallised out, making, with the amount already isolated, a total weight of 1·73 g.

The washings containing the soluble barium carbamate were boiled and the precipitated barium carbonate was filtered off. The filtrate, on evaporation, gave nearly pure alanine. The yield after recrystallisation from aqueous alcohol was 14·04 g. (N: found, 15·83 %; calc., 15·73 %.) In view of the difficulty of effecting a complete separation of alanine and glycine, either by

the ester method or by the formation of their calcium chloride compounds [Pfeiffer and Wittka, 1915] it may be pointed out that the procedure described above gives a very clean and satisfactory separation of these two substances.

Fraction III. Amino-acids from copper salts insoluble in methyl alcohol and water.

The copper salts in this fraction were brought into solution by suspending them in water and adding small quantities of sulphuric acid from time to time as necessary, and were decomposed as before.

The alcohol-insoluble barium salts from this fraction yielded, when decomposed, *aspartic acid.* (Yield after recrystallisation from water 3·7 g., making, in all, 4·22 g. or 1·85 % of the weight of glutenin.)

Leucine and phenylalanine. The main filtrate from the barium aspartate was freed from alcohol and barium and evaporated to a small volume on the water-bath. Most of the material crystallised out in flakes which were filtered off and washed with cold water. On evaporating the mother-liquor and washings a small second crop of crystals was obtained. The dry crystals contained 10·03 % N. The mother-liquor, on evaporation to dryness, gave a crystalline powder, the nitrogen content of which (10·01 %) showed it to be of similar composition to the earlier crops of crystals. The method of fractionation employed suggested that this material was a mixture of leucine and phenylalanine. Separation of the two amino-acids however was only partially successful. By repeated fractional crystallisation 8·5 g. of nearly pure leucine was obtained. (M.P. 280–285°. N: found, 10·4 %; calc., 10·69 %.) The residual mixture (12 g. containing 9·76 % nitrogen) was converted into the zinc salt [Brazier, 1930]. From the water-soluble portion of the dried salts, after removal of zinc, 3·45 g. of crude phenylalanine was obtained. (N: found, 8·86 %; calc., 8·49 %.)

The residue insoluble in cold water was made alkaline with a little barium hydroxide and decomposed with hydrogen sulphide. On evaporating the solution 8·5 g. of material having 10·13 % N crystallised out. As it was found impossible to effect any further separation either by crystallisation or by the use of the zinc salts the phenylalanine content was determined indirectly by oxidation to benzoic acid [Kollmann, 1928]. The mixture was oxidised by boiling for 6 hours with Beckmann's solution; the benzoic acid, together with the fatty acids formed, were extracted in a continuous extractor with ether, and after evaporation of the ether the benzoic acid was separated by washing the residue with water previously saturated with benzoic acid. The benzoic acid was dried to constant weight at 60°. Duplicate experiments gave the following results: 1·505 g. of the mixture yielded 0·3476 g. of benzoic acid, equivalent to 23·33 % of phenylalanine; 1 g. gave 0·1645 g. of benzoic acid equivalent to 23·66 % of phenylalanine. 0·5 g. of pure phenylalanine gave 0·3476 g. of benzoic acid corresponding to a yield of 94 %. The mixture thus contained 23·5 % phenylalanine and 76·5 % leucine. Calculated from the nitrogen

content the figures are 25·5 % and 74·5 % respectively. As the discrepancies are within the limits of error of the oxidation method it seems legitimate to assume that the mixture consisted only of leucine and phenylalanine and to adopt the latter of the above sets of figures as representing its composition. On this assumption the gross value for phenylalanine is 2·75 % and for leucine 6·3 % of the glutenin.

The following tables summarise the results obtained. Table I gives the distribution of nitrogen in the main fractions at the stage prior to the liberation of the amino-acids from their copper salts. Table II gives the percentages of the different products isolated, both in terms of total nitrogen and of total weight of protein taken, together with the values obtained by previous workers for comparison. Column 2 of Table II gives an indication of the losses encountered; those in precipitates are in some cases ascribed to particular amino-acids. It is seen, for example, that the value for glycine is at least 20 %, for alanine 5 % and for aspartic acid 10 % too low. By combining the highest values from the present analysis with those obtained by previous workers 70·98 % of the protein is now accounted for in the products of hydrolysis. In the case of the monoamino-acids, with which this method is chiefly concerned, the value 22·14 % given by the ester method has been raised to 27·34 %.

Table I. *Distribution of nitrogen in different fractions.*

	Nitrogen in g.	Nitrogen %
Humin	0·45	1·17
In main barium sulphate precipitate	2·51	6·46
In solution	35·06	90·36
Loss	0·79	2·01
Total	38·81	100·0
In tyrosine isolated before forming copper salts	0·53	1·36
In ammonia	7·41	19·10
In excess copper carbonate	0·26	0·67
In copper salts	26·53	68·38
Loss	0·33	0·85
Total	35·06	90·36
In Fraction I of the copper salts	4·64	11·96
In Fraction II	17·75	45·74
In Fraction III	3·17	8·17
Loss	0·97	2·51
Total	26·53	68·38

Discussion.

The following advantages can be claimed for the method of separation described.

1. By avoiding the losses inherent in the ester method higher yields of most of the monoamino-acids have been obtained, notably in the case of proline, valine, alanine and phenylalanine.

Table II.

	% of total nitrogen. Copper salt method		% of weight of protein	
	In products isolated	In precipitates and unidentified syrups	Copper salt method	Other methods
Humin	—	1·17	—	—
Main barium sulphate precipitate ...	—	6·46	—	—
Ammonia	19·1	—	3·97	4·01[1]
Excess copper carbonate...	—	0·67	—	—
Tyrosine (both fractions)...	1·91	—	4·20	4·25[1]
Fraction I:				
Copper sulphide	—	0·53	—	—
Valine	0·72	—	1·02	0·2[1]
Proline	4·39	—	6·15	5·98[2]
Unidentified syrup	—	5·91	—	—
Fraction II:				
Copper sulphide	—	1·60	—	—
Barium sulphate from first precipitation	—	0·22	—	—
Glutamic acid	14·78	—	26·49	25·7[3]
Hydroxyglutamic acid	0·5	—	1·00	1·8[4]
Lead hydroxide and sulphate	—	0·07	—	—
Arginine and histidine	11·60	—	—	—
Silver and barium precipitates	—	0·25	—	—
Lysine and unidentified matter... ...	—	6·42	—	—
Barium phosphotungstate	—	0·20	—	—
Glycine (from Fractions I and II) ...	0·83	—	0·76	0·89[1]
Barium sulphate from carbamate ...	—	0·19	—	—
Alanine	5·68	—	6·16	4·65[1]
Barium sulphate	—	0·29	—	—
Fraction III:				
Copper sulphide	—	1·05	—	—
Barium sulphate	—	0·09	—	—
Aspartic acid	1·15	—	1·85	2·0[3]
Leucine	3·96	—	6·3	5·95[1]
Phenylalanine	1·37	—	2·75	1·97[1]

[1] Osborne and Clapp [1906].
[2] Spörer and Kapfhammer [1930].
[3] Jones and Moeller [1928].
[4] Dakin [1919].

2. The indeterminate losses of the ester method are replaced by definite losses in precipitates which can be readily determined. Thus some idea is gained of the deficiencies falling on individual amino-acids.

3. The fractionation of the copper salts gives rise to groups of amino-acids which are more amenable to separation than are the fractions obtained by conversion into the esters. Thus alanine, valine and the leucines, which in the ester method form one group extremely difficult to separate, are here obtained in three fractions.

4. The amino-acids set free from the copper salts are, as a rule, free from contaminating syrupy material and therefore readily crystallisable.

On the other hand, the procedure described here, though adopted after

many preliminary separations on rather different lines, still suffers from the following drawbacks.

(*a*) The considerable loss of the hydrolysis products in the first barium sulphate precipitate.

(*b*) The difficulty of separating the copper salts of aspartic and glutamic acids in the presence of large amounts of the latter.

(*c*) The inability to isolate serine and cystine. In one preliminary experiment the dicarboxylic acids and the diamino-acids were first removed, the former as the barium salts, the latter by means of phosphotungstic acid, prior to conversion into copper salts. This procedure was abandoned on the ground that the barium dicarboxylates carried traces of other amino-acids with them and the basic phosphotungstates were not completely insoluble. Later experience has however shown that the preliminary removal of these two groups of amino-acids would considerably simplify the fractionation of the copper salts of the monoamino-acids and in future work this modification is recommended.

SUMMARY.

1. The hydrolysis products of glutenin have been fractionated by means of their copper salts.
2. Higher yields of many of the monoamino-acids have been obtained.
3. Possible improvements in the method are indicated.

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