# CXXXIII. A NEW METHOD FOR THE SEPARATION OF THE PRODUCTS OF PROTEIN HYDROLYSIS.

By MARY AGNES BURNISTON BRAZIER.

*From the Biochemical Laboratory, Imperial College of Science and Technology, London, S.W. 7.*

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It has long been acknowledged that the methods available for the quantitative separation of the products of hydrolysis of proteins are not entirely satisfactory. The yields of individual amino-acids are often low, and the manipulative processes involved in their separation are difficult. The well-known process, due to Emil Fischer, in which the amino-acids are separated mainly by the fractionation of their esters at very low pressure remains the standard method of separation except in some special cases. Alternative methods have been put forward from time to time, but these have been concerned with particular fractions of the hydrolysis products, and no comprehensive scheme avoiding the use of ester-fractionation has been advanced for the separation of the amino-acids. Among the newer methods of dealing with the mixture of amino-acids may be mentioned the butyl alcohol extraction method of Dakin [1920] and the calcium salt and lead salt methods of Foreman [1919]. The object of all these has been to overcome, in regard to particular details, some of the objections to the ester method of Fischer, but none has entirely dispensed with the use of the esters at some stage of the process.

The older methods enabled the early workers to investigate the hydrolysis products of many proteins with considerable success, but in no case was the complete analysis of a protein possible. A separation which accounted for 75 % of the total nitrogen represented a careful and successful fractionation; more usually little more than 60 % of the nitrogen could be traced. In some of the later hydrolyses about 85 % of the nitrogen was accounted for [Foreman, 1919].

It is evident from the results quoted that a more satisfactory technique was required.

With these considerations in view, much work has been carried out during the past 7 years, under the direction of the late Professor S. B. Schryver, with the object of finding a method for the separation of the amino-acids from among the hydrolysis products of proteins which should fulfil, as far as possible, the following conditions:

(i) the method should be capable of furnishing a maximum yield of pure individual amino-acids;

(ii) the use of the esters should be avoided entirely;

(iii) the method should be as simple as possible.

A technique has been evolved which, although not yet perfect, more nearly fulfils these conditions than any previously available.

The method is due to the late Professor Schryver, who was assisted by a number of workers, including the author, who carried through the hydrolysis of zein described in this paper; this constituting the first example of a complete analysis carried out by the method.

An outline of the method has already been published from this laboratory by Town [1928] to whom much of the technique for separating the amino-acids is due.

The separation is based on the fact that the different amino-acids, and certain of their derivatives, in particular their copper salts, show widely different degrees of solubility in solvents such as water, methyl alcohol and ethyl alcohol. The success of the method depends upon the use of very pure dry solvents and thoroughly dry copper salts.

In drying the copper salts, the only satisfactory drying agent was found to be "absolute" acetone. By its use the copper salts are obtained in the form of a fine dry powder, which readily responds to fractionation by means of the appropriate solvents.

The main fractions so obtained are the following.

A. *Copper salts insoluble in water.*

This fraction contains leucine and phenylalanine together with aspartic acid.

B. *Copper salts soluble in water.*

These salts are further separated by extraction with methyl alcohol, giving:

(i) *Copper salts insoluble in methyl alcohol.*
Alanine, tyrosine, glutamic acid, histidine and arginine (also glycine and bases if present).

(ii) *Copper salts soluble in methyl alcohol.*
Valine, hydroxyvaline, proline and prolylphenylalanine.

The further separation of these amino-acids is described in detail below.

The use of the new technique has made it possible, in the case of zein, to isolate 87·9 % of the nitrogen from the neutralised and filtered hydrolysate (which contains only 87·2 % of the total N of the zein) as amino-acids in the approximately pure form, and, by examination of all the precipitates obtained at various stages, a total of 93·5 % of this nitrogen has been traced.

The analysis by this method has not only accounted for a higher total of nitrogen than earlier analyses, but it has in addition led to the discovery in zein of two constituents hitherto undetected—namely hydroxyvaline and prolylphenylalanine.

## Experimental.

### *Preparation of zein.*

The zein used for hydrolysis was prepared from maize flour by repeated extraction with 73 % alcohol; about 10 extractions were made, the maize being left each time to stand overnight under the alcohol. As 73 % alcohol extracts the colouring matter from maize as well as zein, this may be used as a fair indication of the completion of the extraction. The alcohol-soluble portion was dried, and the colouring matter removed by repeatedly extracting with dry acetone. This not only dissolves the colouring matter, but also absorbs the remaining moisture from the zein, which thus becomes granulated to a fine powder. A 3·4 % yield of zein was obtained by this method from the sample of maize used.

### *Hydrolysis of zein.*

The zein so prepared contained 7·2 % water and 0·3 % ash.

250 g. of this material, equivalent to 231 g. moisture-free and ash-free zein (containing 17·5 % nitrogen) were hydrolysed by boiling with 4 times the bulk of 25 % sulphuric acid under a reflux condenser for 24 hours.

On cooling and filtering the hydrolysis solution, a considerable amount of material was found to be insoluble, 18·8 g. in all, equivalent to 8·1 % by weight of zein. This substance contained 9·5 % of nitrogen, but on extraction with ether and alcohol a large quantity of fat was removed, the remaining material being found to contain 12·1 % nitrogen. It appears to be, most probably, incompletely hydrolysed protein protected from hydrolysis by fat; after removal of the fat this material can be hydrolysed in the usual way.

After filtering off this insoluble substance, the filtrate was neutralised with baryta; the precipitate of barium sulphate was boiled up with water 7 or 8 times to extract as much as possible of the amino-acid solution, as this is where the greatest loss of nitrogen takes place; it was found to be more efficient to dry the precipitate in the oven between each washing and grind it to a powder before extracting with boiling water.

An estimation of the nitrogen in this barium sulphate after washing showed that 3·42 g. had been retained. A large part of this is most probably the humin-nitrogen.

After removal of barium sulphate, the nitrogen of the total filtrate, together with the washings, was found to be 35·33 g. This figure is used throughout this paper as the basis on which the percentages of amino-acids are calculated.

The nitrogen at this stage is accounted for as follows:

Table I.

| | |
|---|---|
| N in insoluble material | 1·78 g. |
| N in barium sulphate precipitate | 3·42 g. |
| N in neutralised hydrolysate | 35·33 g. |
| Total | 40·53 g. |

This table shows the total of 40·53 g. of N from 231 g. protein, equivalent to 17·5 % of N in zein. This confirms the figure obtained for the N content of the ash-free zein.

*Removal of ammonia.*

An estimation of the ammonia present (Fraction C, Table II) was made on an aliquot part of the hydrolysis solution; this gave an equivalent of 7·40 g. N as ammonia, *i.e.* 20·95 % of the total. The ammonia from the whole filtrate was removed by making it just alkaline with baryta and drawing air through for several hours with slight warming of the solution.

*Conversion to copper salts.*

When no further ammonia could be detected the solution was made quantitatively free from barium and sulphuric acid; it was then converted into the copper salts by adding copper carbonate to the boiling solution until there was no further effervescence; after boiling for a short time it was evaporated to dryness with excess of the carbonate; the solid so obtained was extracted with cold water in the mechanical shaker for 1 hour, and the extract again evaporated to dryness with more copper carbonate. This was repeated two or three times to ensure complete formation of the copper salts.

This process gives the first stage of separation into two fractions: copper salts soluble in water, and copper salts insoluble in water.

A. *Copper salts insoluble in water.*

This fraction contained the excess of copper carbonate together with the copper salts of leucine, phenylalanine and aspartic acid. An advantage of the copper-salt method is that the two dicarboxylic acids (glutamic and aspartic acids) appear in different fractions.

The whole material was dissolved in dilute sulphuric acid and the copper removed by hydrogen sulphide; the solution was made alkaline with baryta, the barium sulphate was filtered off, and the barium aspartate precipitated by adding 3 volumes of 95 % alcohol. For this precipitation the concentration was approximately 8 g. N in a litre of the solution, to which 3 litres of alcohol were added (Foreman's [1914] method modified by Dakin [1920]).

The barium aspartate was decomposed by adding sulphuric acid quantitatively, the free acid was evaporated to a syrup and granulated with absolute alcohol.

6·55 g. of crude aspartic acid were obtained containing 10·41 % N; this is therefore approximately pure aspartic acid even before recrystallisation. 0·68 g. N was thus isolated as aspartic acid, *i.e.* 1·92 % of the total N in the $BaSO_4$ filtrate from the hydrolysate.

From the crude amino-acid a sample was prepared by boiling with charcoal and repeated recrystallisation, which gave 10·50 % N (calculated 10·53 %).

The alcoholic filtrate from the barium aspartate was freed from excess baryta by sulphuric acid, the barium sulphate being removed by filtration, and the alcohol distilled off; when neutral the solution was concentrated on a water-bath until leucine began to crystallise out. On cooling, large quantities of very nearly pure leucine (containing 10·61 % N) separated, more crystals being obtained on concentrating further. The remaining solution containing the phenylalanine and the rest of the leucine was converted into the zinc salts. After drying at 110° the zinc salt of leucine becomes quite insoluble in cold water, whereas that of phenylalanine is readily soluble. For the preparation of the zinc salts the solution was made up roughly to 500 cc. (containing approximately 5 g. N); excess of freshly precipitated zinc hydroxide was added to the boiling solution, the whole being kept boiling for half an hour; the precipitate was filtered off and the filtrate again boiled with fresh zinc hydroxide to ensure complete formation of the zinc salts. The filtrate was evaporated to dryness and dried at 110°. The precipitate was also dried at this temperature, and each was then extracted with cold water. The extract was evaporated down and again dried at 110° and extracted with cold water; this was repeated until all redissolved.

The insoluble zinc salt of leucine was decomposed in alkaline solution with hydrogen sulphide and evaporated to dryness. The total weight of crude leucine obtained by direct crystallisation and by decomposition of the zinc salt was 51·43 g., containing in all 5·31 g. N, equivalent to 15·05 % of the total N.

From this a specimen of pure leucine was prepared by recrystallisation from hot water, which contained 10·68 % N (calculated 10·69 %).

The zinc salt of phenylalanine was similarly decomposed, and on evaporation the solution of the free acid gave 15·18 g. of crude phenylalanine containing 8·05 % N, *i.e.* 1·28 g. N in all or 3·62 % of the total N.

On repeated recrystallisation a sample was prepared giving 8·37 % N (calculated 8·49 %).

The phenylalanine was found to be much more difficult to obtain in the pure form than leucine, having a tendency to deliquescence.

B. *Copper salts soluble in water.*

These were evaporated as nearly to dryness as possible, and then granulated by stirring the syrup with dry acetone to absorb the last traces of moisture. The acetone is poured off through a filter, and a further batch added to the copper salts; this is repeated many times until all the moisture has been removed and the copper salts appear as a granular powder, with no trace of stickiness.

The last traces of acetone are removed by drying in a vacuum desiccator, and finally at 110° for half an hour.

The copper salts, when dry, were extracted repeatedly (about 10 times) with dry methyl alcohol in the mechanical shaker, until no more dissolved. (The methyl alcohol extract never becomes quite colourless as some moisture

is absorbed from the air, but when three consecutive extractions gave the same depth of blue, the process was judged to be complete.)

In this way two further fractions were obtained, copper salts soluble, and copper salts insoluble, in methyl alcohol.

B1. *Copper salts insoluble in methyl alcohol.*

This fraction contained the copper salts of alanine, glutamic acid, tyrosine, arginine and histidine (glycine, if present, occurs in this fraction, also bases).

After confirming that all would redissolve in water, the copper was removed by hydrogen sulphide, and the solution evaporated down to about 1500 cc. (containing approximately 11 g. N). It was then made alkaline with baryta, the barium sulphate was filtered off, and on addition of three times the volume of 95 % alcohol barium glutamate was precipitated. (Foreman's method, as used in Fraction A for aspartic acid.)

By carrying the precipitation through twice, a clean granular precipitate of barium glutamate was obtained. This precipitate was quantitatively decomposed with sulphuric acid, the barium sulphate filtered off, and the free acid evaporated to dryness; large quantities of crude glutamic acid crystallised out during this evaporation, and were filtered off, the residue being granulated with alcohol. Before recrystallisation this material contained 9·3 % N (calculated 9·52 %).

The total yield of crude glutamic acid weighed 73 g. and contained 6·78 g. N, equivalent to 19·19 % of the whole.

On boiling up with charcoal and recrystallising from water glutamic acid was prepared containing 9·50 % N.

After the removal of the glutamic acid, the barium salts soluble in alcohol were quantitatively decomposed with sulphuric acid, the barium sulphate was filtered off and the solution of free acids concentrated to boil off the alcohol. On further concentration to a small bulk, crystals of tyrosine separated.

These, without recrystallisation, contained 7·73 % N (calculated 7·74 %) and weighed 5·04 g. containing therefore 0·39 g. N, *i.e.* 1·14 % of the total.

The next stage was the separation of the bases, histidine being first removed by formation of its zinc salt and precipitation with mercuric chloride (Schryver's method).

The filtrate from the tyrosine was converted into the zinc salts by boiling for half-an-hour with excess of freshly precipitated zinc hydroxide, after which the excess hydroxide was filtered off, and a solution of mercuric chloride added carefully until there was no further precipitate. Histidine forms a complex compound which is insoluble in water. This was filtered off and decomposed in acid solution with hydrogen sulphide to precipitate the mercury; the solution was filtered and the chlorine removed by adding silver sulphate; the silver was precipitated by hydrogen sulphide, filtered off, and the filtrate made alkaline with baryta; the zinc was then removed by hydrogen sulphide, the

barium sulphate and zinc sulphide being filtered off together. The filtrate when neutral was concentrated on a water-bath, but did not crystallise; it contained 0·70 g. N, equivalent to 1·98 % as histidine. The solution was evaporated to a thick syrup and granulated with alcohol; this gave a fine white powder. As, however, this gave a nitrogen content low for histidine, it was redissolved, boiled with charcoal and again granulated with alcohol. The nitrogen value was still low—24·86 % (calculated 26·96 %) and a trace of ash was found on incineration. The experiments of other workers in this laboratory indicate that histidine alone is precipitated by this method, which suggests that the material prepared from zein was histidine contaminated with inorganic matter probably brought down from the protein itself. No trace of mercury, zinc, silver or barium could be detected in the ash. Further research is necessary on the histidine separation and is in progress.

The remaining solution containing alanine and arginine was freed from chlorine by silver sulphate, and from mercury, silver and zinc by hydrogen sulphide. The solution was concentrated to about 500 cc. (containing 2·5 g. N) and made 0·5 % acid with sulphuric acid; the arginine was then precipitated as the flavianate by Kossel's and Staudt's method [1926].

A solution of flavianic acid in cold 0·5 % sulphuric acid was added and the whole well shaken. (About 2 g. of flavianic acid are required for every g. of arginine present.)

After standing for 3 days a crystalline precipitate formed, which was filtered off and washed with 0·5 % sulphuric acid. These crystals contained no inorganic matter, and charred at 248°; they were still unmelted at 280°. They gave 13·2 % N (micro-Kjeldahl estimation), which accords with a monoflavianate formula [see Kossel and Gross, 1924]; this value is a little high (calculated 12·1 %) but investigations in this laboratory indicate that a small percentage (about 4 %) of the nitrogen from the flavianate radical yields ammonia in a micro-Kjeldahl estimation.

2·6 g. of arginine monoflavianate were obtained, equivalent to 0·96 g. of arginine and 0·31 g. N, *i.e.* 0·88 % of the total.

No trace of any base other than histidine and arginine could be found in zein.

The remaining solution containing alanine was freed from excess of flavianic acid by continuous extraction with butyl alcohol at 60°; the butyl alcohol extract was extracted with water to ensure complete retention of the alanine in the water-soluble fraction. This was then freed from sulphuric acid and evaporated to a syrup. On granulating with alcohol 12·8 g. of material were obtained, containing 1·68 g. N equivalent to 4·76 % as alanine.

By redissolving this and granulating again with alcohol a sample was prepared which gave 15·18 % N (calculated 15·73 %). A specimen of alanine which was much purer has since been prepared by this method from a subsequent hydrolysis.

B 2. *Copper salts soluble in methyl alcohol.*

The methyl alcohol was removed by distillation and the material granulated with acetone; the acetone was removed in a vacuum desiccator and the salts finally dried at 110° for a short time. After confirming that all would redissolve in water and in methyl alcohol, the copper was precipitated by hydrogen sulphide, and the filtrate containing the free acids was neutralised and evaporated to small bulk.

On standing for some days a white crystalline substance separated out, more being obtained on concentrating further.

On recrystallisation from hot water this material was found to be identical with prolylphenylalanine, a peptide first isolated by Osborne and Clapp [1907] from gliadin, and hitherto undiscovered in the hydrolysis products of zein. The specimen prepared contained 10·67 % N (calculated 10·7 %) all in the non-amino-form.

The carbon and hydrogen content of this material agreed with the formula for prolylphenylalanine and derivatives were prepared which confirmed this[1].

2·96 g. of the pure peptide separated at this stage, a further yield being obtained from the valine fraction (see below), giving in all 3·25 g. containing 0·35 g. N, *i.e.* 0·99 % of the total.

The filtrate, from which the peptide had been removed, was evaporated to a syrup and granulated with acetone. The free acids were then extracted with dry absolute alcohol, a large portion remaining insoluble; the soluble portion was repeatedly evaporated to dryness and extracted with alcohol, until all redissolved.

B 2. (i) *Free acids soluble in absolute alcohol.*

The solution contained approximately 5 g. N, almost entirely in the non-amino-form. As this fraction was expected to contain the proline, the whole material was dissolved in water and converted into the picrate by Town's method [1928]. Picric acid was added to the boiling solution in sufficient quantity to combine with the non-amino-nitrogen present; on cooling, crystals of proline picrate together with excess picric acid came down; these were filtered off, and on concentrating further more crystals separated. The whole yield was extracted with ether to dissolve excess picric acid (no ether-soluble picrate was found). On recrystallisation from hot water this material gave the characteristic needle-like crystals of proline picrate melting at 148°.

A further yield of proline was obtained from the mother-liquor by precipitation with cadmium chloride. The mother-liquor from the separated picrate would not crystallise any further, so it was decomposed with 1 % sulphuric acid, and extracted with ether to remove the picric acid.

[1] Micro-combustion of this specimen gave a value of 63·8 % C and 7·29 % H (calculated 64 % C, 6·88 % H).

The experimental results are the mean of several estimations.

The aqueous solution was neutralised, evaporated to dryness, and taken up with absolute alcohol; a trace would not redissolve and was added to the main bulk of alcohol-insoluble acids.

A solution of cadmium chloride in 90 % alcohol was added drop by drop to the alcohol extract until no further precipitate formed; this end-point must be carefully watched as the cadmium-proline compound redissolves in excess cadmium chloride. Town found that proline alone is precipitated at this stage.

The precipitate was filtered off and dissolved in water; it was freed from chlorine by precipitation with silver sulphate, the silver and cadmium being removed together by hydrogen sulphide. After neutralising and filtering, the proline in solution was again converted into the picrate, the crystals so obtained being added to the first yield. In all 53·9 g. of proline picrate were prepared, being equivalent to 18·0 g. of proline and 2·19 % N, *i.e.* 6·2 %.

The filtrate, from which the cadmium-proline compound had been separated, was concentrated to remove the alcohol, and taken up with water. The chlorine was removed by silver sulphate and the silver and cadmium by hydrogen sulphide as before. When quantitatively neutral the solution was evaporated to dryness and taken up with absolute alcohol; a small portion did not redissolve and was added to the main bulk of alcohol-insoluble acids.

The alcohol-soluble fraction contained 2·42 g. N (6·85 %); this nitrogen was almost entirely in the non-amino-form.

This fraction was not examined by the author, but was handed to Professor Schryver. In the corresponding fraction from gliadin, Town has succeeded in isolating two piperazine derivatives, *iso*leucine, and some leucine and tyrosine.

B 2. (ii) *Free acids insoluble in absolute alcohol.*

These were redissolved in water, a small portion remaining insoluble; this proved to be prolylphenylalanine, and was added to the main yield of this peptide (see above).

The aqueous solution was reconverted into copper salts to confirm that all would redissolve in methyl alcohol. A small fraction would not redissolve and was filtered off; this precipitate contained 0·02 g. N.

The methyl alcohol was distilled off from the soluble portion, which was then decomposed with hydrogen sulphide, neutralised and evaporated to dryness. The free acids contained 11·6 % N and were obviously a mixture. They were therefore converted into zinc salts, which were dried at 110° and extracted with absolute alcohol [Schryver and Buston, 1926], the extract being evaporated to dryness and taken up with alcohol several times to ensure complete separation.

The greater portion was insoluble, and on decomposition in alkaline solution with hydrogen sulphide, and after neutralising and evaporating, a syrup was obtained which on repeated granulation with alcohol gave 9·15 g. of valine containing 1·01 g. N, *i.e.* 2·86 %.

From this a sample was prepared by repeatedly redissolving and granulating with alcohol, which gave 11·6 % N (calculated 11·96 %).

The zinc salt soluble in absolute alcohol was freed from alcohol by distillation, dissolved in water and decomposed in a similar manner. The free acid on recrystallisation contained 10·51 % N, all in the amino-form, and was found to be identical with hydroxyvaline (10·53 % N)—first isolated from oat protein by Schryver and Buston [1926].

The carbon and hydrogen contents[1] agreed with those of hydroxyvaline and the material gave similar derivatives (*e.g.* benzoyl derivative M.P. 117°, phenyl *iso*cyanate derivative M.P. 145°).

The total yield of hydroxyvaline was 5·04 g. containing 10·51 % N, *i.e.* 0·53 g. N, which is equivalent to 1·5 % in the form of hydroxyvaline; this being the first time it has been isolated from zein.

This completed the separation of the hydrolysis products of zein, which are represented in the following table by their nitrogen contents. The results of the analysis detailed below are in good agreement with those from several previous hydrolyses of zein carried through by the author in the development of the technique.

Table II.

Total nitrogen to be accounted for = 35·33 g.

| | | Amino-acid | N isolated g. | N as percentage of total N |
|---|---|---|---|---|
| A. | | Aspartic acid | 0·68 | 1·92 |
| | | Leucine | 5·31 | 15·05 |
| | | Phenylalanine | 1·28 | 3·62 |
| B 1. | | Glutamic acid | 6·78 | 19·19 |
| | | Tyrosine | 0·39 | 1·14 |
| | | Histidine | 0·70 | 1·98 |
| | | Arginine | 0·31 | 0·88 |
| | | Alanine | 1·68 | 4·76 |
| B 2. | (i) | Prolylphenylalanine | 0·35 | 0·99 |
| | | Proline | 2·19 | 6·20 |
| | | Alcohol-soluble fraction | 2·42 | 6·85 |
| | (ii) | Valine | 1·01 | 2·86 |
| | | Hydroxyvaline | 0·53 | 1·50 |
| C. | | Ammonia | 7·40 | 20·95 |
| | | Total | 31·03 | 87·89 |

The total shows 87·89 % of the nitrogen as amino-acids isolated in an approximately pure form; 5·6 % more of the nitrogen was found in precipitates, 93·5 % in all being traced.

SUMMARY.

A simple method is described for the analysis of proteins. The separation is based on the different solubilities of the copper salts of the amino-acids in various solvents.

Experimental details are given of a complete analysis of zein carried through by the process. The results show a marked improvement on those of

[1] Micro-combustion of this specimen gave 44·7 % C and 7·95 % H (average of several determinations). $C_5H_{11}O_3N$ requires 45·1 % C and 8·27 % H.

other methods. In the case of zein 93·5 % of the nitrogen of the hydrolysate was traced.

The author owes much to the late Professor Schryver under whose direction most of the work was done.

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