LII. QUANTITATIVE ESTIMATION OF ASPARTIC AND GLUTAMINIC ACIDS IN THE PRODUCTS OF PROTEIN HYDROLYSIS.

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(*Received July 28th, 1914.*)

The figures obtained by the majority of workers when estimating glutaminic acid in caseinogen by separating the hydrochloride in the usual way, are uniformly about 11 per cent. In two cases, however, very much higher yields of the recrystallised hydrochloride have resulted. Thus Osborne and Guest [1911] found 15·55 per cent., and Foreman 15·9 per cent. (unpublished result). In these two cases the most favourable conditions for the separation were obtained, but could not be clearly defined. The accuracy of the results, however, still remained in doubt, and the method is unreliable.

The ease with which the glutaminic acid hydrochloride separates seems to vary with the protein under treatment and with the amount of glutaminic acid present. Plimmer [1912] states that separation of the hydrochloride "occurs in the case of caseinogen and certain vegetable proteins which contain from 10–40 per cent. of this amino-acid." If the protein contains under 10 per cent., then, as a rule, no hydrochloride will separate. Occasionally, however, a separation is obtained; thus Hopkins and Savory [1911] working with Bence-Jones protein obtained 8·05 per cent.

When a protein contains so little glutaminic acid that separation as the hydrochloride before esterification is impossible, or any other difficulties are encountered, the estimation of this amino-acid must depend upon the working up of the higher boiling esters. The yield of glutaminic acid hydrochloride obtained in this way is always very poor when compared with that obtained by separation before esterification. An investigation of the unesterified portion will show that glutaminic and aspartic acids, probably

owing to their dibasic character, do not esterify so readily. From a quantitative standpoint, therefore, at any rate, the percentage of glutaminic acid obtained by working up the higher boiling esters appears to be valueless.

A direct method for the estimation of these two amino-acids is described below.

It seemed possible that a method might be based on some essential difference between the two types—dibasic and monobasic amino-acids. As it is well known that the calcium salts of non-nitrogenous dibasic organic acids have a much higher degree of insolubility in water or alcohol than the calcium salts of non-nitrogenous monobasic acids, it was thought that the same principle might apply in the case of the dibasic and monobasic amino-acids. Experiments were therefore made with a view to testing this matter.

Calcium chloride solution was added to a solution of the amino-acids obtained by the hydrolysis of caseinogen. No precipitate resulted, and on adding much alcohol only a small precipitate was obtained. When, however, another portion of the same solution was made alkaline with lime, a copious precipitate appeared on the addition of alcohol. The precipitate seemed to increase somewhat in quantity when the free ammonia was removed before the alcohol was added.

Solutions of all the monamino-acids found in proteins, with the exception of oxyproline, were then treated separately with lime and alcohol. Glutaminic and aspartic acids, cystine and tyrosine all gave calcium salts insoluble in alcohol. The calcium salts of the other monamino-acids, however, were found to be very soluble in alcohol, and the solutions all remained perfectly clear when the alcohol was added. Pyrrolidonecarboxylic acid has since been tried in the same way, and a copious precipitate was obtained.

Since these observations were made I have found that Abderhalden and Kautzsch [1910] have made the calcium salts of aspartic, glutaminic, and pyrrolidonecarboxylic acids separately from the pure substances in a similar way. They have not suggested, however, that any quantitative use could be made of these facts in reference to separations from hydrolytic products derived from proteins.

The precipitate obtained, by means of lime and alcohol, from the products of the hydrochloric acid hydrolysis of caseinogen, has been found to consist of the calcium compounds of the following substances:

(*a*) glutaminic and aspartic acids;

(*b*) a gummy substance very difficult to crystallise;

(*c*) a very small quantity of a pigmented substance precipitated from the cold aqueous solution by silver sulphate solution;

(*d*) a substance precipitated by phosphotungstic acid.

The calcium salt of tyrosine separates from the alcoholic filtrate on standing a long time.

The phosphotungstic precipitate, derived in the manner stated, from 20 g. caseinogen gave 0·57 g. dry substance after removing the phosphotungstic acid. An aqueous solution of this substance was neutralised with nitric acid and silver nitrate solution added. No precipitate resulted. On adding ammonia, however, a precipitate was obtained. This dissolved in excess. The further investigation of this substance is postponed.

Description of the Method.

20–40 g. of the protein are hydrolysed by boiling for 48 hours with three times the quantity of hydrochloric acid, and the liquid evaporated to a syrup at 45° and 15 mm., thus removing the large excess of hydrochloric acid.

Formation of the calcium salts and removal of the humins. The syrup is taken up in 200–400 cc. water, depending upon the quantity of protein taken. Pure calcium oxide is slaked, made into a cream with water, thoroughly cooled, and added to the liquid in sufficient quantity to ensure a good excess. As a rule, 0·5 g. calcium oxide to every gram of protein appears to be required. The flask is corked, and the cold liquid shaken for a few minutes and then filtered on a Buchner funnel, and the residue thoroughly washed with cold water. The insoluble part contains the humin bodies and the excess of lime.

Removal of excess of water and ammonia. The slightly coloured filtrate is evaporated at 40–45° and 15 mm., so that the remaining liquid will possess a volume corresponding to 3·5–4 cc. for each gram of protein taken. This operation also serves to remove all traces of ammonia which can be estimated if desired. As I have shown elsewhere [Foreman, 1914] glutaminic acid is converted into pyrrolidonecarboxylic acid to a considerable extent when its solution is boiled with excess of lime. No such change, however, occurs at 40–45°. The temperature is therefore not allowed to exceed this limit. The tube admitting air into the distilling flask should be fitted with a soda lime tube to prevent the entrance of carbon-dioxide.

Precipitation of the calcium salts insoluble in alcohol. Rectified spirit (97 per cent. alcohol) is now added to the solution in the distilling flask in small quantities at a time with vigorous shaking. The alcohol should be

added gradually, in order to prevent the precipitate forming a clot which would tend to carry down other substances. The first part of the precipitate is inclined to be sticky, and if the alcohol be gradually added with shaking, this part adheres to the sides of the flask as a thin uniform layer which can be afterwards easily washed. The addition of the alcohol is continued until further precipitation ceases. About a litre of alcohol is required as a rule.

The precipitate is now filtered on a Buchner funnel and the flask with its adhering layer as well as the contents of the funnel washed thoroughly with rectified spirit. Occasionally when the alcoholic washings enter the clear main filtrate, an opalescence appears on standing, and an insignificant amount of precipitate may separate. When this is filtered off further similar separations may be obtained in a like manner. These small precipitates contain no glutaminic or aspartic acids and may be neglected. The calcium salt of tyrosine separates slowly from such a mixture in this manner.

Quantitative removal of the calcium from the alcohol-insoluble calcium salts. The precipitate is taken up in about 300 cc. water and the calcium quantitatively removed by means of oxalic acid.

Removal of traces of chloride and a pigmented substance precipitated by cold silver sulphate solution. The calcium oxalate is filtered off and aqueous silver sulphate solution added to the cooled liquid until no further precipitate results. This removes a small amount of chloride and also a very small quantity of a peculiar pigmented substance, the precipitate presenting a flocculent appearance. If the liquid be heated on the water bath before filtering, the silver compound of the pigmented substance dissolves but the substance is precipitated by phosphotungstic acid, and is included in the precipitate obtained later on.

The slight excess of silver is now removed from the solution by means of sulphuretted hydrogen.

The slightly coloured filtrate from the silver sulphide is reduced to about half its bulk on the water bath to remove sulphuretted hydrogen.

Removal of substance precipitated by phosphotungstic acid. An aqueous solution of phosphotungstic acid (Merck's) is then added until the colour disappears from the solution, 5–10 g. being usually sufficient. The liquid at this stage will continue to yield insignificant traces of precipitate if more of the phosphotungstic acid solution be added, and about as much again is required in order to carry the precipitation to absolute completion. This further addition of phosphotungstic acid is unnecessary, however, as the following test shows.

A small test portion of the liquid is taken after the colour has disappeared, and made up so as to contain 5 per cent. sulphuric acid. To this is added a few drops of a 25 per cent. solution of phosphotungstic acid in 5 per cent. sulphuric acid. No precipitate results. By adding no more phosphotungstic acid after the disappearance of the colour, one avoids a much larger precipitate of barium phosphotungstate to be obtained on removing the excess, and possible loss of the amino-acids by occlusion is thus reduced to a minimum. The usual method of precipitation in 5 per cent. sulphuric acid was avoided, as in this case the presence of the acid is unnecessary, and loss by occlusion in a large barium sulphate precipitate, produced on its removal, is prevented.

Baryta water is now added to the solution at 45° until no further precipitate results. If the solution at this stage possesses a blue colour[1], the addition of baryta water should be continued until the colour completely disappears.

The small precipitate of barium phosphotungstate is filtered off, and the slight excess of baryta quantitatively removed by means of sulphuric acid.

Separation of the monamino-acids in the dry state. The small barium sulphate precipitate is filtered off, and the liquid evaporated to about 50 cc. on the water bath. It is then transferred to a weighed dish, containing a weighed stirring rod, and slowly evaporated to 20–25 cc. (corresponding to 40 g. protein taken originally) on a warm water bath. No trace of tyrosine appears. The amino-acids crystallise on cooling, and the semi-solid mass is stirred and placed in a vacuum desiccator over calcium chloride over night. By the next morning the amino-acids will have dried to hard gritty cakes. These can be broken up with the rod and placed for a further period in the vacuum desiccator, but as a rule the loss of weight in this second period is very small indeed.

Separation of the glutaminic and aspartic acids by extraction with cold glacial acetic acid in which these acids remain undissolved. The dry substance is then triturated with cold glacial acetic acid in the dish by means of a small glass pestle, and filtered on a small Buchner funnel. The glutaminic and aspartic acids remain undissolved. The solid is again placed in the dish and extracted in the same way with more cold glacial acetic acid. It is finally all transferred to the filter and washed two or three times with glacial acetic acid. The white solid, which comes off the filter paper quite cleanly, is transferred to a weighed dish, dried and weighed.

[1] A blue colouration appeared in the filtrate when using Kahlbaum's phosphotungstic acid. This did not occur when Merck's product was employed.

Treatment of the acetic acid extract. The extract yields a gum on removing the acetic acid. It is placed in a weighed porcelain dish and the acetic acid removed as completely as possible *in vacuo* at 40–45°.

The remaining traces of acetic acid can be removed in a vacuum desiccator over strong potash. Further drying may be carried out *in vacuo* over calcium chloride if considered necessary.

A further trace, usually under 0·1 g., may be obtained by extracting this dry residue a second time.

Estimation of pyrrolidonecarboxylic acid, present in the substance soluble in cold glacial acetic acid.

The moist gum is now weighed quickly and thoroughly mixed by stirring with a strong glass rod. The rod is then used to transfer a 0·2 g. portion to a weighing tube for an amino-nitrogen determination. Without delay a further 0·5 g. portion is weighed into a bulb of 4–5 cc. capacity, and about 2 cc. strong hydrochloric acid added. A long tube is then sealed to the stem of the bulb to act as a reflux condenser. The bulb is allowed to rest lightly on a sand bath, and the liquid boiled for about 12 hours. The liquid is then made up to 20 cc. with water, and the amino-nitrogen determined in a portion of this solution. The difference between the amino-nitrogen present before and after boiling with hydrochloric acid represents pyrrolidonecarboxylic acid. The total amount of this acid present in the moist gum can then be found, and its equivalent of glutaminic acid calculated. This is added to the weight of glutaminic acid separated as such, before calculating the percentage in the protein.

If confirmation be required, the remainder of the moist gum may be extracted with cold absolute alcohol in which pyrrolidonecarboxylic acid is soluble. The alcohol can then be evaporated as completely as possible from this extract in a weighed dish and the weight of this alcohol-soluble substance obtained. The amino-nitrogen can then be determined in portions weighed out, without delay, before and after boiling with strong hydrochloric acid in the manner already described.

Instead of weighing out portions of the gum it would have been easier to have made its aqueous solution up to a known volume. This procedure, however, was not adopted because of the difficulty of transferring the gum, derived from the evaporation of an aliquot portion, to the bulb for boiling with hydrochloric acid.

Estimation of the proportion of glutaminic acid to aspartic acid in the mixture of these two acids obtained.

The white substance, which was insoluble in cold glacial acetic acid, is then submitted to analysis. The proportion of glutaminic acid to aspartic acid is calculated on the carbon content of the mixture.

As the differences between the percentages of carbon in glutaminic and aspartic acids is as great as 4·73 the proportion can be determined with tolerable accuracy.

The purity of the mixture, and the proportions calculated from the carbon content can be checked by a nitrogen determination.

Separation of the two constituents of the mixture for further identification.

A known weight of the mixture is converted into the copper salts in plenty of water, and the filtered liquid allowed to cool. If crystals of copper aspartate do not separate after standing some time, the liquid is gently reduced in bulk until signs of crystallisation appear. After standing over night the light blue needles are filtered off, and an attempt may be made to obtain a further crop from the mother liquor in the same way. As a general rule, however, the first crop will contain practically all the aspartic acid. At any rate, this has been shown to be true when the aspartic acid is present to a greater extent in the mixture than the glutaminic acid. Occasionally, if allowed to stand too long, the characteristic small heavy dark blue prisms of copper glutaminate may appear mixed with the light blue needles. These crystals, however, can be separated quite easily from the needles by elutriation with cold water.

The filtrate from the copper aspartate is freed from copper by means of hydrogen sulphide and the liquid evaporated to dryness in a weighed dish. After taking the weight of the dry substance hydrochloric acid is added in the proportion of 1·8 to 2 cc. for each g. substance. The liquid is boiled for a short time, cooled and saturated with dry hydrochloric acid gas in the usual way. The glutaminic acid hydrochloride thus obtained can be weighed and analysed.

A maximum separation of glutaminic acid hydrochloride appears to result when the substance and the hydrochloric acid are taken in the proportion stated.

Should copper aspartate refuse to separate through being present in the mixture in too small a proportion, the glutaminic acid must be separated first as the hydrochloride. The filtrate from this is then evaporated to dryness and the copper aspartate may be obtained by working up this residue.

Results Obtained by Applying the Method to the Products of the Hydrolysis of Caseinogen.

The method as above described was applied to 40 g. "Hammarsten casein" containing 14·08 per cent. nitrogen and 9·3 per cent. moisture. Calculating on the basis that pure caseinogen contains 15·62 per cent. nitrogen, the 40 g. is equal to 36·05 g. of the pure substance. The hydrolysis was carried out by boiling with three times the quantity of hydrochloric acid (sp. gr. 1·16) for 48 hours. The following results were obtained:

Total weight of dry substance before extraction with the cold glacial acetic acid		13·48 g.
Weight of glutaminic and aspartic acids insoluble in the cold glacial acetic acid, dried to constant weight at 100°		
The first extraction	6·996 g.	
The second extraction	·025 g.	7·021 g.
Difference = weight of dry gum		6·46 g.

Analysis of the mixture of glutaminic and aspartic acids.

The white substance weighing 7·02 g. dried to constant weight at 100° gave the following results on analysis:

0·1684 g.; 0·2494 g. CO_2; 0·0902 g. H_2O.

0·1550 g.; 12·8 cc. nitrogen at 20° and 768 mm. by Dumas' method.

0·1634 g.; 27·0 cc. amino-nitrogen at 20° and 768 mm. by van Slyke's method.

The percentages calculated from these results are shown below side by side with the theoretical percentages present in glutaminic and aspartic acids:

	Calculated for glutaminic acid	Found	Calculated for aspartic acid
Carbon	40·82	40·39	36·09
Hydrogen	6·12	5·95	5·26
Nitrogen	9·52	9·57	10·52
Amino-nitrogen ..	9·52	9·56	10·52

The proportion of aspartic acid to glutaminic acid in the mixture is therefore shown as follows:

$$\frac{40{\cdot}82 - 40{\cdot}39}{40{\cdot}82 - 36{\cdot}09} = \frac{1}{11};$$

$$\frac{1}{11} \text{ of } 7{\cdot}02 = 0{\cdot}64 \text{ g. aspartic acid,}$$

$$\frac{10}{11} \text{ of } 7{\cdot}02 = 6{\cdot}38 \text{ g. glutaminic acid.}$$

Estimation of pyrrolidonecarboxylic acid in the gummy substance soluble in the cold glacial acetic acid.

Practically the whole of the 0·025 g. substance obtained by the second extraction came from the acetic washings of the 7 g. Through determining this fact two lots of gum were obtained:

A. from the main filtrate;

B. from the washings.

These were treated separately, as it was afterwards found inconvenient to mix them.

When dried, as far as possible in the manner described, the two portions of the moist gum weighed as follows:

A. 6·29 g.
B. 2·48 g. } equal to 6·46 g. in the dry state.

The amino-nitrogen was then determined before and after boiling for 16 hours with hydrochloric acid, with the following results:

A. 0·161 g. gave 13·8 cc. amino-nitrogen at 20·8° and 768 mm. 0·4542 g. was boiled with 2·5 cc. strong hydrochloric acid for the time stated, and then made up to 20 cc. 10 cc. of this gave 27·1 cc. amino-nitrogen at 24·3° and 762 mm.

B. 0·2100 g. gave 19·2 cc. amino-nitrogen at 19° and 771 mm. 0·4705 g. was treated with hydrochloric acid in exactly the same way as *A*, and then made up to 20 cc. 8·6 cc. of this gave 22·9 cc. amino-nitrogen at 23° and 763 mm.

From these figures the following results were calculated:

Weight of moist gum	Percentage of amino-nitrogen before boiling with HCl	Percentage after boiling with HCl	Difference
A. 6·29 g.	4·94	6·70	1·76
B. 2·48 „	5·24	6·41	1·17

Calculating the increase in amino-nitrogen into terms of glutaminic acid, it will be found that 1·163 g. is thus obtained from *A* and 0·305 g. from *B*, making a total of 1·468 g.

In order to prove that this increase in amino-nitrogen was due to the hydrolysis of pyrrolidonecarboxylic acid, the remaining gummy substance was extracted with cold absolute alcohol, which dissolves this acid, and the extract was boiled for 10 hours with hydrochloric acid after removing the alcohol. The brown solution, now containing glutaminic acid, was warmed

with animal charcoal and filtered. The filtrate was then evaporated to about 5 cc. and saturated with hydrochloric acid gas at 0°. The perfectly white glutaminic acid hydrochloride which separated, was then obtained in the dry state in the ordinary way, and weighed. After allowing for the portions of the original gummy substance previously removed 0·993 g. of the hydrochloride resulted.

As stated elsewhere, it is very probable that a small quantity of the pyrrolidonecarboxylic acid is decomposed when boiled with acid, thus explaining the brown colour which invariably appears. Other slight errors were introduced in the alcoholic extraction and the animal charcoal treatment. Taking these facts into consideration, the yield is consistent with that usually obtained by the hydrochloride method.

The glutaminic acid hydrochloride melted at 201–202°.

The following results were obtained on analysis :

0·1481 g. ; 0·1593 g. CO_2 ; 0·0675 g. H_2O.

0·1529 g. gave by Dumas' method 9·1 cc. nitrogen at 18·5° and 753 mm.

0·1009 g. ; 0·0091 g., equal to 9·02 per cent. of ash.

Allowing for the ash, the following figures were calculated :

	Found per cent.	Calculated for glutaminic acid hydrochloride per cent.
Carbon	32·25	32·69
Hydrogen	5·56	5·45
Nitrogen	7·52	7·62

It is difficult to account for the ash unless one assumes that it was derived from the phosphotungstic acid used early in the method.

The products of the hydrolysis of the 36·05 g. pure caseinogen therefore contained :

1·468 + 6·38 = 7·848 g. glutaminic acid,
0·64 ,, aspartic acid.

The percentages in the protein work out as follows :

21·77 per cent. glutaminic acid,
1·77 ,, ,, aspartic acid.

Results of a previous trial with caseinogen.

For this trial, 20 g. " Hammarsten casein " equal to 18 g. of the pure substance were taken. The calcium salts were precipitated by alcohol in two fractions. Before adding the alcohol the volume was reduced at 40–45° and 15 mm. to about 100 cc. In the 40 g. trial, already described, the volume

was about 120 cc. before adding the alcohol, so that relatively more water was present when precipitating the calcium salts in this 20 g. trial.

The two fractions were obtained as follows. The alcohol was added in small quantities at a time with shaking until very little colour remained in the solution. The second fraction was obtained by completing the precipitation with more alcohol. For convenience these two lots of precipitate may be labelled *A* and *B*.

These two fractions *A* and *B* were worked up, in the way already described, omitting all precautions as to temperature. Finally the substance, instead of being dried in the desiccator before extracting with acetic acid, was heated in the dish on the water bath with occasional stirring until dry. The cakes were then crushed and dried to constant weight in the steam oven. The acetic acid was removed from the extracts on the water bath, and the gummy residues were afterwards dried to constant weight in the steam oven before analysis.

The following weights of dry substance resulted:

	Weight of dry substance before extraction with acetic acid	Weight of dry substance insoluble in acetic acid	Difference; weight of dry gum
Fraction *A*. ..	2·06 g.	1·129 g.	0·93 g.
„ *B*. ..	3·28 „	1·638 „	1·64 „
Total ..	5·34 „	2·767 „	2·57 „

Second extractions of the two gummy portions with cold glacial acetic acid yielded:

Fraction *A*. 0·081 g.
„ *B*. 0·077 „

The acetic acid was removed from these extracts on the water bath and the gums were dried in the steam oven. Portions were then taken, dried to constant weight, and analysed.

The following results were obtained:

	Fraction *A*; insoluble in acetic acid	Fraction *B*; insoluble in acetic acid	Fraction *A*. The dry gum soluble in acetic acid	Fraction *B*. The dry gum soluble in acetic acid
Carbon	40·08	40·51	45·45	46·04
Hydrogen	6·33	6·68	6·0	6·1
Amino-nitrogen ..	9·84	9·36	1·97	2·55
Kjeldahl nitrogen ..			11·26	11·31

Pyrrolidonecarboxylic acid contains:

Carbon	46·51 per cent.
Hydrogen	5·43 „ „
Nitrogen	10·85 „ „
Amino-nitrogen	nil

These two portions of gum contain smaller percentages of amino-nitrogen than was found in the gum from the 40 g. caseinogen trial. The total weight of dry substance before extraction with the acetic acid is also relatively less. Thus from the 20 g. caseinogen 5·34 g. were obtained and from the 40 g. caseinogen 13·48 g., giving a difference of 2·8 g. per 40 g. caseinogen. On calculating, it will be found that this 2·8 g. contains about 10 per cent. amino-nitrogen. By allowing less water to be present when precipitating the calcium salts with alcohol, 2·8 g. of some substance soluble in cold glacial acetic acid containing about 10 per cent. amino-nitrogen has therefore been obtained in addition. The identity of this constituent of the gum has not yet been established but the matter is receiving attention.

It will be noted that the analyses of the gums do not differ widely from the calculated figures for pyrrolidonecarboxylic acid. The total nitrogen is about 0·4 per cent. higher than the total nitrogen of this acid. If basic lead acetate solution be added to a portion of the aqueous solution of the gums a large precipitate, characteristic of pyrrolidonecarboxylic acid, results on adding plenty of alcohol. If this precipitate be filtered off and extracted with cold water, a coloured lead compound remains undissolved. On decomposing this with sulphuretted hydrogen a small quantity of brown substance (0·145 g. from 20 g. caseinogen) is obtained on evaporation. This body probably possesses a high nitrogen content, but has so far received no further attention.

In order to show more clearly that the increase in amino-nitrogen which is always obtained by boiling the gum with hydrochloric acid is due to the hydrolysis of pyrrolidonecarboxylic acid, the remaining gum was extracted with cold absolute alcohol, which dissolves pyrrolidonecarboxylic acid. The alcohol was removed from the extract, and the amino-nitrogen was determined before and after boiling with hydrochloric acid. It was argued that a much greater increase in the amino-nitrogen in this alcohol-soluble substance would give further satisfactory evidence on this point.

0·7808 g. of the dry gum was allowed to remain in contact with cold absolute alcohol for some time, in a desiccator to prevent absorption of moisture, stirring occasionally with a strong glass rod. The solution became slightly coloured, and the insoluble part gradually appeared in a powdery form. The liquid was filtered and the extraction completed with fresh cold alcohol. The alcohol was then completely removed in a vacuum desiccator over sulphuric acid, yielding 0·6267 of moist gum. Three portions of this were weighed out quickly, one for a moisture determination, and the other two

for amino-nitrogen determinations before and after boiling with strong hydrochloric acid.

0·1465 g. lost 0·0295 g. when dried at 100° till its weight was constant. The gum therefore contained 20·13 per cent. moisture, and the 0·6267 g. contained 0·5014 g. dry substance.

0·1420 g. of the moist gum gave by the nitrous acid method 4·25 cc. amino-nitrogen at 21° and 768 mm. The moist gum therefore contained 1·72 per cent. amino-nitrogen.

0·3261 g. was boiled with 1·6 cc. strong hydrochloric acid in a small bulb as before described, for 20 hours. The liquid was then made up to 20 cc. and the amino-nitrogen determined in 10 cc. of this solution. 24·2 cc. amino-nitrogen at 24·6° and 763 mm. were obtained. The percentage of amino-nitrogen calculated on the weight of moist gum taken was therefore 8·29.

By boiling with the acid the amino-nitrogen had therefore increased from 1·72 per cent. to 8·29 per cent.

Expressing this increase in terms of pyrrolidonecarboxylic acid hydrolysed by the acid, the 0·5014 g. of dry alcohol-soluble substance contained 0·3795 g. of this acid. As this 0·3795 g. was present in 0·7808 g. dry gum, 1·25 g. would be contained in the whole 2·57 g. dry gum obtained from the 18 g. pure caseinogen. This 1·25 g. corresponds to 1·42 g. glutaminic acid.

As the 0·5014 g. alcohol-soluble substance contains 0·3795 g. pyrrolidone carboxylic acid, the difference, 0·1219 g. contains the amino-nitrogen present before boiling with acid. It will be found by calculating, that this 0·1219 g. contained 8·8 per cent. amino-nitrogen. The figure should probably be nearer 10 per cent., as on boiling pure pyrrolidonecarboxylic acid with strong hydrochloric acid, 94 per cent. of change is generally accounted for, the remaining 6 per cent. probably decomposing to produce something giving the brown colour which always appears.

The portion of the 0·7808 g. gum insoluble in the alcohol amounting to 0·2704 g. and equal to 4·9 per cent. of the caseinogen should contain no amino-nitrogen. It gave a dark blue copper salt insoluble in alcohol which showed no tendency to crystallise. The investigation of this substance is proceeding.

The total weight of glutaminic and aspartic acids thus accounted for in this 20 g. caseinogen trial is shown as follows:

	Weight in g.	Percentage of the protein
Separated by means of the glacial acetic acid ..	2·925	16·22
From the pyrrolidonecarboxylic acid	1·42	7·88
	4·345	24·1

The total percentage of glutaminic and aspartic acids in the protein obtained in the 40 g. caseinogen trial was 23·54. The results obtained in the two trials therefore practically correspond.

In order to compare the two results the figures are shown side by side, as follows:

		Per cent. separated as glutaminic and aspartic acids	Per cent. glutaminic acid, estimated from the pyrrolidonecarboxylic acid content of the gum	Total per cent.
Trial *A* ..	20 g. caseinogen	16·22	7·88	24·1
Trial *B* ..	40 g. caseinogen	19·47	4·07	23·54[1]

It will be noted that in trial *A*, the amount of pyrrolidonecarboxylic acid present in the gum was greater than in trial *B*. The amount of glutaminic and aspartic acids separated as such, however, is correspondingly less. When carrying out trial *A*, however, no temperature precautions in the final stages were taken, as already explained. In another paper it will be shown that glutaminic acid changes into *l*-pyrrolidonecarboxylic acid when its aqueous solution is boiled. The change also occurs, but to a much smaller extent, at the temperature obtained in a liquid contained in an open dish on the boiling water bath. It has also been shown that even strong acids like hydrochloric and sulphuric do not completely inhibit the change until they are present to the extent of 3 per cent. and 8 per cent. respectively. These facts were not determined at the time trial *B* was carried out, the only information available at that time being that a change took place when an aqueous solution of glutaminic acid was heated with lime. The temperature precautions taken in the final stages of trial *B* were instinctive. The inference is obvious that these precautions account for the higher yield of glutaminic and the corresponding lower yield of pyrrolidonecarboxylic acid.

When carrying out trial *B* the liquid from time to time was frequently evaporated, and sometimes heated in an open dish or in a flask on the water bath, which will account for the conversion of glutaminic acid into pyrrolidonecarboxylic acid to the extent of 4·07 per cent. of the protein. As it has also been shown in another place that *l*-pyrrolidonecarboxylic acid is hydrolysed to glutaminic acid by boiling for 4 hours with strong hydrochloric acid, it is very difficult to conceive of the pyrrolidonecarboxylic acid required by this 4·07 per cent. glutaminic acid existing in the original hydrolytic liquid obtained by boiling the caseinogen for 48 hours with strong hydrochloric acid. Its formation from glutaminic acid, afterwards, appears to need no further discussion.

[1] I consider 23·54 the more accurate figure.

The Method applied to the Soluble Protein of the Swede Turnip.

This work was carried out by G. Williams, who has given me permission to refer to his results, which are awaiting publication. He was unable to obtain any glutaminic acid as the hydrochloride before esterification. In working through the ester process he obtained 2·75 per cent. aspartic and 0·26 per cent. glutaminic acid.

Using the new method he obtained 6·98 per cent. aspartic acid and 3·18 per cent. glutaminic acid, without working up the gummy substance containing pyrrolidonecarboxylic acid which it has been shown can be formed from glutaminic acid during the operations. The percentage of glutaminic acid will be increased when this gum has been worked up.

The glutaminic and aspartic acid mixture insoluble in the cold glacial acetic acid, gave the following analysis shown side by side with the calculated percentages in the pure ingredients:

	Calculated for aspartic acid per cent.	Found in the mixture per cent.	Calculated for glutaminic acid
Carbon	36·09	37·44	40·81
Hydrogen ..	5·27	5·67	6·12
Nitrogen ..	10·52	10·18	9·52

The method already described for separating the two constituents of the mixture was afterwards applied and practically all the aspartic acid was obtained as copper aspartate and nearly all the glutaminic acid partly as copper glutaminate, separated by elutriation, and the remainder as the hydrochloride from the mother liquor from the copper salts. These products all gave very satisfactory analyses.

Experiments to show that the Calcium Salts of Glutaminic and Aspartic Acids can be Precipitated Quantitatively by Means of Alcohol.

1 g. pure glutaminic acid and 1 g. pure aspartic acid were dissolved separately in about 20 cc. water, and these solutions were treated in exactly the same way. Pure calcium oxide previously made into the form of a cream with water, and cooled, was added until present in excess. The liquids were then reduced to 4 cc. at 40–45° and 15 mm. and rectified spirit added until precipitation ceased. The precipitates were filtered off and thoroughly

washed with alcohol. The filtrate and washings in each case were combined and the nitrogen content of the whole liquid determined by Kjeldahl's method. The same amount of N/10 acid, viz. 0·15 cc., was neutralised by the ammonia in each case. 99·78 per cent. of the glutaminic acid and 99·8 per cent. of the aspartic acid therefore had been precipitated under these conditions.

In a previous experiment, using 1 g. glutaminic acid, the volume of the aqueous solution of calcium glutaminate was 30–40 cc. before adding the alcohol; 83 per cent. was precipitated, determined in the way described. The amount of water present before adding the alcohol is therefore a factor that decides the yield.

It has been shown that practically the same percentages of glutaminic and aspartic acids were obtained in the 20 g. and 40 g. caseinogen trials. In the 20 g. trial, however, the volume of the aqueous solution of the calcium salts before adding the alcohol was in the proportion of 4 to 5 cc. for each gram of caseinogen originally taken, whilst in the 40 g. trial it was 3 to 3·5 cc. It therefore appears that the yield of glutaminic and aspartic acids is not affected if these limits be observed when separating them from proteins by this method. When the smaller quantity of water is present, however, more amino-nitrogen is present in the glacial acetic acid extract.

Additional evidence for the quantitative character of the precipitation of the calcium salts of the two dibasic acids was obtained by working up the alcoholic filtrate from the 40 g. caseinogen trial. The alcohol was removed, and the remaining liquid reduced to a smaller bulk than for the first precipitation. Alcohol was again added and a very small amount of precipitate, which took a long time to settle, resulted. This was filtered off, and two further small quantities obtained in succession by repeating this whole operation. These three precipitates were combined, and worked up in the same way as the main precipitate. No traces of glutaminic and aspartic acids, however, were found. The aqueous solution obtained at the finish which should have contained these acids if they had been present was neutral to litmus even when highly concentrated, and the small quantity insoluble in cold glacial acetic acid was found to consist of tyrosine. It is therefore claimed that all the glutaminic and aspartic acids were present in the main precipitate.

SUMMARY.

1. The calcium salts of glutaminic and aspartic acids are precipitated quantitatively from their aqueous solutions by means of alcohol, provided that these solutions are sufficiently concentrated.

2. These calcium salts are also precipitated quantitatively in the same manner, from a solution containing the calcium salts of the amino-acids resulting from the acid hydrolysis of proteins.

3. Glutaminic and aspartic acids are practically insoluble in cold glacial acetic acid, and this fact is made use of in separating them from the other products obtained.

Pyrrolidonecarboxylic acid was present in the acetic acid extract.

4. Evidence is given here, as well as in another paper [1914], to show that glutaminic acid is converted into pyrrolidonecarboxylic acid to some extent during the operations, through exposing the solutions from time to time to temperatures in the neighbourhood of 80–90°.

This pyrrolidonecarboxylic acid can be reconverted into glutaminic acid by boiling with hydrochloric acid, and the degree of change from ring nitrogen to the amino-form enables an estimation to be made.

5. Other unidentified substances probably of great importance are obtained by the method.

6. The application of the method to two proteins has given substantially greater yields of glutaminic and aspartic acids than obtained by the older methods, and it is believed that the results are quantitative.

The method applies equally to proteins which contain small percentages of either of the two amino-acids, and only a small quantity of protein is needed.

ADDENDUM.

The foregoing description applies to the method as it has been used up to the present. Subsequent work, published in another paper [1914], has shown that at such temperatures as the solutions attained when heated on the water bath from time to time during the operations, glutaminic acid is transformed to some extent into pyrrolidonecarboxylic acid. Evidence has already been given to show that the pyrrolidonecarboxylic acid found when applying the method to caseinogen was formed in this way. A further trial is therefore in progress wherein the temperature at any stage of the

operations will not be allowed to exceed 45°. There can be very little doubt that these extra precautions will result in the isolation of the whole of the glutaminic acid as such.

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