CCII. THE ISOLATION OF GLUTAMINE FROM AN ENZYMIC DIGEST OF GLIADIN.

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The recent isolation of asparagine from an enzymic digest of edestin [Damodaran, 1932] provided the first direct evidence for the existence of a dicarboxylic acid amide in the protein molecule. As was pointed out in that paper it is, perhaps, from the point of view of demonstrating the validity of the amide hypothesis more important to isolate glutamine, because the majority of proteins on acid hydrolysis yield much larger amounts of glutamic acid than aspartic acid. But glutamine was known to be unstable in aqueous solution [Chibnall and Westall, 1932] so that at the end of the period required for the digestion of a protein to the amino-acid stage only a portion of the glutamine set free would still be present as such in the digest. Furthermore, the work of Schulze on the isolation of glutamine from plant extracts suggested that the separation of this amide from the digest would be difficult, and it was for these two reasons that the isolation of asparagine, which is stable in aqueous solution, and which crystallises readily in the presence of other amino-acids, was first attempted. With the experience thus gained a successful attempt has now been made to isolate glutamine from an enzymic digest of gliadin. This protein was chosen because of the high proportion of glutamic acid which it gives on acid hydrolysis; in terms of total protein-N it contains 25·7 % of amide-N, 22·9 % of glutamic acid-N, 1·17 % of hydroxyglutamic acid-N, and only 0·5 % of aspartic acid-N. In other words, if the validity of the peptide and amide hypotheses be assumed the gliadin molecule should yield 42·2 % of glutamine.

At the outset an unforeseen difficulty arose, in that this prolamine was not as susceptible to the action of proteolytic enzymes as, for example, the edestin used in the asparagine research. While with this, and many other of the better known proteins, the successive action of pepsin, trypsin and either intestinal erepsin or yeast dipeptidase could accomplish the liberation of 90 % of the amino-N obtained on complete hydrolysis [Damodaran, 1932; Frankel, 1916], with gliadin it was found that the same enzymes split little more than 70 % of the total peptide bonds. The phenomenon, which invites further

investigation, is undoubtedly connected with peculiarities of molecular structure which must be responsible for the distinctive behaviour of the prolamines as a class and no appreciable improvement in the degree of hydrolysis was attained by variations in the conditions of digestion such as changes in p_H or previous denaturation of the protein.

Since the deficiency in action fell specially on pepsin, which produced a liberation of only about 10–12 % of peptide bonds compared with the 20 % obtained with edestin, it was thought that it might with advantage be replaced or reinforced by a protease of vegetable origin. Papain was accordingly tried, both instead of and in succession to pepsin. No use could, however, be made of the definitely larger hydrolysis thus obtained, as it was found to be accompanied by a simultaneous liberation of large quantities of ammonia, showing that the enzyme preparation was contaminated with an amidase which hydrolysed the amide group of the glutamine. It was necessary, therefore, to use the same three enzymes which had been employed in the digestion of edestin regardless of the much smaller hydrolysis effected.

In carrying out the digestion the procedure adopted followed closely that of the previous investigation, with such small modifications as had been suggested by previous experience or were called for on account of the difference in properties between glutamine and asparagine. In the former research, for instance, the optimum period for the action of each enzyme could be readily ascertained from determinations of amino-N and free ammonia-N. In the present case the instability of glutamine in solution suggested the advisability of determining also the glutamine amide-N. As will be seen from the tables given later this precaution was fully justified, because in the later stages of the digestion, when splitting of the peptide bonds was approaching a maximum, the rate of decomposition of the glutamine already present in the digest exceeded that at which glutamine was being set free from amino-complexes.

The determination of glutamine by this indirect method was also necessary for another purpose, *viz.* the calculation of the percentage hydrolysis at different stages. The usual procedure is to compare the Van Slyke amino-N present in the digest at any stage with the total amino-N obtained on complete hydrolysis. But as was first clearly pointed out by Chibnall and Westall, glutamine behaves abnormally with nitrous acid, both amino- and amide-groups being decomposed, giving nearly twice the theoretical amino-N value. Therefore, in a digest rich in glutamine, such as would be obtained from gliadin, the Van Slyke amino-N values cannot be accepted as a true measure of the extent of the peptide splitting. An approximate value for this can, however, be obtained by deducting an amount equal to the glutamine amide-N present, as determined by the method of Chibnall and Westall [1932]. It is to be remembered that these workers intended this method, which is based on the rapid liberation of ammonia from glutamine in solution when heated at 100° for 3 hours at either p_H 4 or p_H 8, to be used primarily for the determination of the glutamine content of plant extracts, from which glutamine

peptides have not yet been isolated. They showed, however, that synthetic glutamine peptides of the type

$$R.CH(NH_2).CO.NH.CH(CH_2.CH_2.CO.NH_2).CO_2H$$

were stable. Glutamine peptides of the type

$$R.CH(CO_2H).NH.CO.CH(NH_2).CH_2.CH_2.CONH_2,$$

however, have not yet been synthesised, so that their stability is not known, but as the glutamine amino-group, which is in the γ-position to the amide group, is free, it is possible that they may yield ammonia under the prescribed conditions, and also give abnormal values for Van Slyke amino-N. Now it is probable that peptides of both these types are present in an enzymic digest of gliadin, so that the glutamic amide-N and corrected amino-N values quoted in this paper can have only approximate significance.

The digest of 150 g. of gliadin, from which glutamine was finally isolated, contained initially 27·07 g. of N. At the end of the digestion period the corrected amino-N value was only 69·7 % of that given on acid hydrolysis, so that more than 30 % of the peptide bonds were still intact. Of the amide-N given on complete hydrolysis 31·1 % was present as free ammonia-N and 42·9 % as glutamine amide-N, so that about 25 % was still present in stable amides or peptide amides. Assuming that the glutamine amide-N had its origin in glutamine the amount of the latter present in the digest was 28·1 g.

After treatment with phosphotungstic acid and removal of reagents the clarified solution contained only 6·7 g. of N. Allowing for the basic-N content of the protein (10 % of the total N) and the free ammonia-N (7·5 % of the total N) it is clear that the major part of the residual peptide bonds must have been present in compounds predominantly basic in character, which have been precipitated by this reagent. The calculated amount of glutamine in the clarified solution mentioned above was 10·1 g. As the material recovered from the phosphotungstic acid precipitate contained only a relatively small amount of glutamine amide-N, it appears that the major part of the lost glutamine must have been decomposed during the precipitation operations. The clarified solution gave on treatment 65·8 g. of crystalline material, and after intensive fractional crystallisation 3·4 g. of pure glutamine were finally isolated. This yield, though disappointing as a method of preparing glutamine, must be considered satisfactory when it is remembered that only about one-third of the protein taken was present in the solution after removal of the more complex constituents and that by the end of the digestion nearly one-third of the amide-N was converted into free ammonia. The 3·4 g. of material isolated compares not unfavourably with the 10 g. of glutamine indicated in the final solution by the indirect method, considering the difficulties of crystallising glutamine from a mixture of amino-acids of varying degrees of solubility.

With the existing technique it would be impossible either to prepare an enzymic digest of a protein in which all the asparagine and glutamine originally

present in the protein was liberated intact, or to obtain anything approaching a quantitative isolation of these two amides from an amino-acid mixture, so that the question whether the amide groups of dicarboxylic acids constitute the sole source of the ammonia obtained from proteins must still remain open. But attempts to verify, by actual isolation, the existence of alternative sources of ammonia such as the uramino-acids suggested by Lippich and the ureide groups of Schutzenberger have already been made without success. The negative results of these investigations seem indeed to have been one of the reasons for the general acceptance of the amide hypothesis.

Although the existence of amide groups has been tacitly assumed for a long time, little recognition has ever been given to the practical consequences of such an assumption, mainly it seems because of the lack of any direct evidence. Thus in numerous investigations on the enzymic digestion of proteins the amount of ammonia formed has been used, on the one hand, as a measure of the deamidising activity of the tissue preparations or enzymes used [Luck, 1924, 1; Hunter and Smith, 1925], and, on the other hand, as a basis of conclusions on protein structure [Luck, 1924, 2; Clementi, 1931], without taking cognizance of the fact that the ammonia might have its origin not in enzymic activity but in the secondary decomposition of any glutamine present, either during digestion or even in the course of the actual determination of ammonia. It is interesting to note that the data in the literature on the deamidising activity of different tissues is extremely conflicting, and also that Luck and Clementi, using the same kind of evidence, came to exactly opposite conclusions as to the source of ammonia in proteins. The possibility is also worth considering that the extreme discrepancies attaching to the determinations of blood-ammonia may be connected with the presence in blood of glutamine, which may be absorbed into the blood-stream from the intestinal tract along with other amino-acids.

It will also be apparent that in determining the degree of hydrolysis in protein digests the values obtained from Van Slyke amino-N determinations are bound to disagree with those based on other methods, such as titrations or conductivity measurements, and that in investigations of the kinetics of proteolysis or of the equivalence of amino-groups and carboxyl-groups the conclusions will be of little value unless account can be taken of the discrepancies introduced by the presence of glutamine or labile glutamine peptides.

Experimental.

The gliadin was prepared by extraction of wheat gluten with 73 % alcohol in the usual way and pouring the alcoholic solution after concentration *in vacuo* into acetone, when the protein was precipitated as a fine powder without undergoing any denaturation; moisture, 10 %; N (moisture-free), 17·3 %. The enzyme preparations were similar to those used in the previous work [Damodaran, 1932]. The papain employed in some of the preliminary experiments was obtained from Merck.

Ammonia- and amino-N were determined by the methods employed by Damodaran [1932], while glutamine amide-N was determined after heating at p_H 4 for 3 hours by the method of Chibnall and Westall [1932]. As glutamine was shown by the latter workers to give 92 % of the total N as amino-N in the Van Slyke apparatus, the necessary correction has been made in computing the amino-N value of certain of the digests.

Preliminary digestion experiments.

Degree of hydrolysis attainable. The preliminary experiments were made with 5 g. samples of gliadin in a similar way to those previously carried out with edestin [Damodaran, 1932]. A slightly different procedure was, however, adopted to bring the protein into solution in the first instance. The 5 g. of gliadin were suspended in about 200 cc. $N/20$ HCl, or in later experiments sulphuric acid, and 0·25 g. pepsin dissolved in 25 cc. $N/20$ acid added. The mixture was incubated at 37° and when the solution had attained homogeneity (usually after about 18 hours) the volume was made up to 250 cc. at 37°.

Table I summarises the results of a typical experiment.

Table I. *Showing the digestion of gliadin by pepsin, trypsin and yeast-dipeptidase.*

Enzyme	Duration of digestion (days)	Amino-N (% total amino-N)	Ammonia-N (% total amide-N)	Glutamine amide-N (% total amide-N)
Pepsin-HCl	2	10·3	7·8	—
followed by Trypsin	3	42·6	14·5	22·5
followed by Yeast-dipeptidase	3	63·8	22·2	33·3
„	4	71·7	26·7	31·1

It will be seen that on prolonging the digestion in the last stage from 3 to 4 days, though there is an increase in the amino-N liberated, the glutamine amide-N has fallen from 33·3 to 31·1 %.

Effect of using sulphuric acid and baryta in place of hydrochloric acid and sodium hydroxide. The peptic digest with sulphuric acid was obtained in the same way as with hydrochloric acid. Since the little evidence available in the literature concerning the influence of barium ions on tryptic action is of a conflicting nature it was thought advisable to add baryta only to neutrality, remove the barium sulphate and then bring the solution to p_H 8·5 by the addition of sodium hydroxide, thus avoiding the presence of barium in solution. The results obtained showed little significant difference from those already recorded with hydrochloric acid and in all subsequent digests sulphuric acid was therefore used for securing the necessary acidity for peptic action and most of the acid removed by means of baryta before the addition of trypsin. Determinations on the loss of material during precipitation with baryta showed that after two washings in the cold the barium sulphate contained less than 6 % of the total N in the solution.

Digestion with papain. As papain used directly was found to act but slowly on gliadin, the latter was first brought into solution through the action of pepsin in the usual way, the p_H adjusted to 5 by the addition of sodium hydroxide and a solution of Merck's papain then added. When the peptide splitting with papain had reached a maximum the digestion was continued with trypsin in the usual way.

The results (Table II) would indicate that papain either contains an amidase or has itself a deamidising action and is therefore unsuitable for the purposes of the present research.

Table II. *Showing the digestion of gliadin with pepsin, papain and trypsin.*

Enzyme	Duration (days)	Amino-N (% total amino-N)	Ammonia-N (% of total amide-N)	Glutamine amide-N (% of total amide-N)
Pepsin, followed by papain followed by Trypsin	2½ 3	16·3 44·2	19·7 31·9	Nil Nil

Preparation of a large-scale digest.

150 g. of gliadin and 7·5 g. of pepsin were dissolved in 7·5 litres of 0·05 *N* sulphuric acid at 37°. After 1 day's digestion a further 5 g. of pepsin, dissolved in 25 cc. of the acid, was added. Analysis of an aliquot showed that the digest contained 25·05 g. of N, and after complete hydrolysis 14·97 g. of amino-N and 6·38 g. of amide-N. Additional aliquots were removed from time to time and analysed for free ammonia-N, amino-N and glutamine amide-N, as shown in Table III.

Table III. *Showing extent of hydrolysis in the large-scale digest.*

Enzyme	Duration of digestion (hours)	Amino-N (% total amino-N) Directly determined	Amino-N (% total amino-N) Corrected for glutamine present	Ammonia-N (% amide-N)	Glutamine amide-N (% amide-N)
Pepsin	17	9·5	9·5	4·1	Nil
,,	65	10·1	10·1	10·7	Nil
Trypsin	63	54·5	50·1	16·3	20·8
,,	87	60·2	52·0	18·8	24·1
,,	101	55·2	51·1	21·7	19·9
Yeast-dipeptidase	43	73·6	60·4	27·1	44·0
,,	91	82·5	69·7	31·1	42·9

After 65 days' peptic digestion baryta in the form of a thin cream was added to p_H 7, the precipitate of barium sulphate centrifuged off and washed twice with water. The clear liquid and washings were brought to p_H 8·5 by the addition of *N* NaOH and 10 g. of trypsin in aqueous solution together with 30 cc. of tricresol added. The reaction of the solution was checked daily, and *N* NaOH added to maintain the p_H at 8·5. At the end of 2 days a further 5·0 g. of trypsin was added. Tryptic digestion was allowed to proceed 5 days

in all, when the solution (8600 cc.) was brought back to p_H 7·5 by the addition of sulphuric acid. 500 cc. of a preparation of yeast-dipeptidase were then added, followed by a similar quantity the following day. After 71 hours it was found that the glutamine amide-N had reached a maximum of about 43 % of the total amide-N. The 9600 cc. of digest therefore contained at this stage roughly 28·1 g. of glutamine.

Fractionation of the large-scale digest.

The digest was rapidly concentrated *in vacuo* at 40° to 2200 cc. Two aliquots of 100 cc. were then removed to find out the approximate amount of phosphotungstic acid required for complete precipitation. These trials were deemed necessary because a previous large-scale digest had shown that at this stage nearly two-thirds of the nitrogen in solution was precipitated by this reagent, and we were desirous of finding out if this unexpectedly large proportion was due in part to nitrogen occluded or adsorbed on the precipitate. The two precipitates formed in the present case, after thorough washing with the usual phosphotungstic acid wash-solution, were found to have carried down 64 % of the total N, whereas in the previous experiment, in which the washing was not so thorough, the amount was 67 %. There seemed to be no doubt, therefore, that the digests still contained a large proportion of peptides *etc.* which were predominantly basic in character.

In carrying out this operation on the main fraction of the digest it was necessary to bear in mind the great instability of glutamine at the strongly acid and alkaline reactions involved in the precipitation with phosphotungstic acid and the subsequent removal of excess of this reagent with baryta. The 2 litres of solution were therefore divided into four 500 cc. portions, each portion being submitted to all the operations involved as quickly as possible, brought to neutrality and allowed to stand in the refrigerator before the next 500 cc. portion was dealt with. As the precipitates had perforce to be allowed to stand some time before being washed, with possible decomposition of glutamine, the washings from the different precipitates were not combined with the main solution until after analysis. The exact procedure was as follows. 500 cc. of the solution were cooled in ice and 25 % sulphuric acid, also previously cooled in ice, added to a concentration of 4 % by volume. The solution was allowed to regain room temperature and a concentrated solution of phosphotungstic acid added until precipitation was complete. The mixture was centrifuged, the clear liquid was made strongly alkaline with a thin cream of baryta to precipitate excess phosphotungstic acid and again centrifuged. The solution was brought to p_H 7 with sulphuric acid and allowed to stand in the refrigerator while the same operations were carried out in succession on the remaining three portions of the digest. The neutral solutions were then combined and the precipitated barium sulphate was centrifuged off.

The precipitate of barium phosphotungstate was now washed with saturated baryta, the washings made neutral with sulphuric acid, the barium

Table IV. *Showing the results of complete hydrolysis of the large-scale digest at different stages.*

Digest	Total N (g.)	Amino-N		Amide-N	
		Weight (g.)	% total N	Weight (g.)	% total N
Peptic	25·05	14·97	59·8	6·38	26·7
Tryptic	26·55	15·17	57·2	6·23	23·4
Yeast	27·07	17·54	64·8	6·23	23·0

sulphate centrifuged off and washed twice with cold water. This solution was combined with similar washings of the main barium sulphate precipitate.

The main solution and the collected washings were then separately concentrated *in vacuo* until tyrosine and other sparingly soluble material began to crystallise out. The two solutions were allowed to stand overnight at 0°, filtered, and made up to 250 cc. and 100 cc. respectively. The analyses of these two solutions are given in Table V. It will be seen that they contained but little free ammonia, and about the same proportion of glutamine amide-N. The total glutamine amide-N was 0·967 g., equivalent to 10·07 g. of glutamine.

Table V. *Analysis of the main solution and the washings after treatment with phosphotungstic acid and crystallisation of sparingly soluble material.*

	Main solution (250 cc.)		Washings (100 cc.)	
	Weight (g.)	% total N	Weight (g.)	% total N
Total N	4·999	—	1·729	—
Ammonia-N	0·047	0·94	0·036	2·1
Glutamine amide-N	0·747	14·95	0·220	12·7
Amino-N	3·866	77·34	Not determined	

Isolation of glutamine.

The solution and washings were combined, rapidly evaporated *in vacuo* at 40°, and the material separating out collected in four successive fractions. After the removal of the fourth crop of crystals the solution had attained the consistency of a thin syrup and no separation of material took place on standing overnight in the refrigerator. As it was not advisable in the present case to allow the solution to stand for the long periods that had been permissible in the previous work on asparagine, alcohol was made use of at this stage to help crystallisation. The solution was warmed on a water-bath to about 70° and 95 % alcohol added till a slight turbidity was produced (about 2 vols. of alcohol were required). The solution was clarified by further warming, and on standing at 0° overnight separation of crystalline material took place. After filtration the solution was again concentrated *in vacuo* at 40° and the treatment with alcohol repeated. Three fairly large fractions thus separated were in the form of white crystalline powders and were easily obtained dry and free from syrupy material by washing with absolute alcohol and leaving in the vacuum desiccator. The eighth fraction was a buttery solid capable of being dried by repeated washing with alcohol, but hygroscopic. The ninth fraction was obtained only on the addition of a large excess of alcohol and

could not be completely dehydrated even with acetone. A last fraction was obtained by evaporating the alcoholic mother-liquors and repeatedly dehydrating the syrup thus obtained with acetone.

The analyses of the various fractions are given in Table VI.

Table VI. *Analysis of various crystal fractions.*

Fraction	Weight (g.)	Total N %	Glutamine amide-N (% total N)
I	1·2	10·6	3·0
II	7·0	10·4	3·7
III	10·4	12·2	13·2
IV	10·0	11·4	14·8
V	9·5	3·3	27·9
VI	4·4	12·4	28·9
VII	9·0	12·0	14·9
VIII	9·2	12·7	13·4
IX	3·0	11·7	9·3
X	2·1	9·1	5·9

It will be recalled that from the corresponding digest of edestin the asparagine, which is much less soluble in water than the majority of monoamino-acids, and which crystallises readily in their presence, crystallised out early in the fractionation in one well-defined fraction. As was to be expected from the greater solubility of glutamine in water, this amide was, in the present case, fairly evenly distributed among a number of the fractions. A much more laborious fractionation was accordingly found to be necessary before glutamine could be isolated.

Fractions I and II could be neglected as they contained but little amide-N; while fraction V consisted almost entirely of inorganic material. Both on account of their syrupy nature as well as the comparatively small amount of material present in them, fractions IX and X were also excluded. Fractions III and IV, being similar to each other, were combined, as also were fractions VII and VIII. The two combined fractions thus obtained as well as fraction VI were then submitted to a series of fractional crystallisations. The characteristic needle-like appearance of glutamine crystals under the microscope, together with the analytical data for total N and amide-N obtained for each separate fraction aided the separation of the glutamine from the other amino-acids. The richest fractions obtained from the three sources were then combined and recrystallised from water. 3·6 g. of nearly pure glutamine were thus obtained (N = 18·8 %). One further recrystallisation from water gave 3·4 g. of pure glutamine. For analysis it was dried *in vacuo* over phosphorus pentoxide. (Found: C, 41·3; H, 6·9; N, 19·2 %. $C_5H_{10}O_3N_2$ requires C, 41·1; H, 6·9; N, 19·2 %.) The amide-N (Sachsse) was 50·2 % of the total N. For a 3·6 % solution in water the observed rotation was $\alpha_D^{22^\circ} + 0{\cdot}35^\circ$, whence $[\alpha]_D^{22} = +5^\circ$; Schulze and Trier [1912] found for their purest preparations of glutamine from plant sources $[\alpha]_D + 6^\circ$ to $+ 7^\circ$.

SUMMARY.

Glutamine has been isolated from an enzymic digest of gliadin, thereby providing additional evidence of the validity of the amide hypothesis, which was first demonstrated by the isolation of asparagine from edestin.

The instability of glutamine in aqueous solution, and its bearing on previous work on the nature and extent of enzymic hydrolysis of proteins are briefly discussed.

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