LXXXIV. THE HOFMANN DEGRADATION OF GLUTAMINE RESIDUES IN GLIADIN

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THE question of the mode of linkage of the ammonia liberated in the hydrolysis of proteins was reviewed by Damodaran [1932] in his paper describing the isolation of asparagine from an enzymic digest of edestin. This work provided the first direct evidence for the long-standing hypothesis that the ammonia so liberated has its origin in the hydrolysis of glutamine and asparagine residues. Shortly after this, Damodaran *et al.* [1932] announced the isolation of glutamine from an enzymic digest of gliadin. In the course of digestion and isolation large losses occurred which were estimated by the method of Chibnall & Westall [1932]; in this way the amount of free glutamine (and, as Melville [1935] has shown, of glutaminyl peptides) present at each stage was determined. The liberation of ammonia in the enzymic digestion of proteins has since been more closely investigated by Damodaran & Ananta-Narayanan [1938] and Damodaran & Narayanan [1938], who have shown that although pepsin and trypsin do not appear to hydrolyse amides set free in the course of digestion, partial hydrolysis of these is brought about by the acid and alkaline conditions necessary for the action of the enzymes.

There is strong evidence, from the work of Damodaran *et al.* [1932] that a much higher proportion of the glutamic acid of gliadin is present in the form of glutamine residues than the amount isolated by them directly as glutamine; the "indirect" method of Chibnall & Westall [1932] showed that at one stage in digestion the "glutamine amide-N" had risen to 44% of the "total amide-N". But since the enzymic method of isolation is known to involve large losses due both to the incompleteness of enzymic hydrolysis of the protein and to secondary decomposition of glutamine and glutaminyl peptides, with liberation of ammonia, it seems that some technique other than the use of enzymic hydrolysis is necessary in order to allocate more definitely the ammonia liberated in protein hydrolysis.

It would be particularly desirable if asparagine and glutamine residues in protein could, before hydrolysis, be converted into residues stable to prolonged boiling with acid, and of such a character that they could be isolated with ease from an acid hydrolysate.

The treatment which immediately suggests itself is the Hofmann degradation of amides by alkali and bromine to the next lower primary amine; in this degradation glutamine would become $\alpha\gamma$-diaminobutyric acid:

$$HOOC.CHNH_2.CH_2.CH_2.CONH_2 \rightarrow HOOC.CHNH_2.CH_2.CH_2.NH_2,$$

and similarly asparagine would become $\alpha\beta$-diaminopropionic acid.

An attempt to degrade free asparagine in this manner, and thus to determine the position of its amide group, was made by Van Dam [1897] without success. Karrer and associates more recently carried out the reaction on *N*-acetylasparagine [1923] and *N*-acetylglutamine [1926]; in the degradation of the latter Karrer obtained a yield of 18% of *l*(+)-$\alpha\gamma$-diaminobutyric acid. Kanewskaja

[1936] applied the Hofmann degradation to a number of benzamido-acid amides, and obtained good yields of the resulting diamines. She stated that where the benzamido group was in the β position to the —$CONH_2$ group, but not in other positions, a glyoxalidone derivative resulted, with elimination of the benzoyl residue. This generalizes the discovery of Karrer & Schlosser [1923] that *N*-acetylasparagine on treatment with alkali and bromine gives glyoxalidonecarboxylic acid, which on acid hydrolysis yields $\alpha\beta$-diaminopropionic acid.

In the present work it was found that, under the conditions used by Kanewskaja, the yield of *l*(+)-$\alpha\gamma$-diaminobutyric acid from *N*-acetylglutamine was about 50 %. The procedure finally adopted for isolation of the base was to precipitate it, after acid hydrolysis, with phosphotungstic acid. (Fischer [1901] and Karrer *et al.* [1926] report respectively that *dl*- and *l*(+)-$\alpha\gamma$-diaminobutyric acids give a precipitate with phosphotungstic acid in the cold.) The phosphotungstic acid precipitate was decomposed with baryta, Ba was removed from the resulting solution with excess of H_2SO_4, and the base, on addition of flavianic acid, crystallized out as the sparingly soluble diflavianate.

In view of the favourable yield of degradation product obtainable from *N*-acetylglutamine, it was decided to apply the procedure to a protein, and subsequently to attempt to isolate *l*(+)-$\alpha\gamma$-diaminobutyric acid from its acid hydrolysate. Wheat gliadin was chosen for its high glutamic acid and low aspartic acid content, and its content of ammonia equivalent to the glutamic acid present. Gliadin also appeared particularly favourable on account of its low content of basic amino-acids [Osborne *et al.* 1915].

It was thought that the phosphotungstic acid precipitate from the acid hydrolysate of gliadin which had been treated with alkaline hypobromite could be decomposed, and arginine and histidine, if present, removed from the filtrate by the silver-baryta precipitation of Kossel, since this procedure was found not to precipitate *l*(+)-$\alpha\gamma$-diaminobutyric acid. It was later found that the silver-baryta precipitation could be omitted, since it only precipitated a small percentage of the amino-N resulting from the decomposition of the phosphotungstic acid precipitate. In the final procedure, *l*(+)-$\alpha\gamma$-diaminobutyric acid was isolated directly as the diflavianate after decomposition of the phosphotungstic acid precipitate. It was identified with the product from glutamine.

Under the best conditions found, with correction for the minimum losses occurring in the course of isolation, a yield of base was obtained, corresponding to 14–16·5 % of the glutamic acid resulting from acid hydrolysis of the protein. This figure is based on an assumed glutamic acid figure for gliadin of 43 % [Jones & Wilson, 1928]. The corresponding figure for glutamine isolated by Damodaran *et al.* [1932] is 5·6 % of the glutamic acid.

The figure given here must represent a lower limit for the percentage of glutamic acid present in gliadin as glutamine, but it gives no indication of an upper limit, since when *N*-acetylglutamine was added to the gliadin solution immediately before degradation the *additional* *l*(+)-$\alpha\gamma$-diaminobutyric acid recovered corresponded to a yield of only 10–38 % from the added *N*-acetylglutamine. Skraup [1907] reported that treatment of casein with alkaline hypobromite in the cold greatly diminished the amount of amino-acids obtainable on hydrolysis, giving rise, among other products, to fatty acids and aldehydes. He found the yield of lysine and, surprisingly, histidine to be undiminished, although arginine was completely destroyed. He was unable to isolate glutamic acid from the hydrolysate, using the technique of direct precipitation as the hydrochloride. The present work suggests that most of the arginine and histidine present in the gliadin are destroyed by treatment with

hypobromite at 80°. Goldschmidt and associates [1925–33] have studied the action of cold hypobromite on amino-acids, peptides and proteins, and have suggested a mechanism to explain how tri- and tetra-peptides give rise to nitriles, keto-acids and amino-acids. At the higher temperature employed in the present work considerable destruction of amino-acids certainly resulted. The number of possible side-reactions under these conditions is so large that the low yield of diaminobutyric acid from *N*-acetylglutamine when degraded in the presence of protein is not surprising.

In view of this, no estimate can be made of the yield from the glutamine residues of the protein on degradation, but since acetylglutamine alone gives a yield of about 50%, the yield from these is probably less.

An attempt to increase the yield of diaminobutyric acid by using a peptic digest of gliadin which had been acetylated with a view to stabilizing free amino-groups against attack by alkaline hypobromite was unsuccessful; the yield of base isolated after acid hydrolysis was about the same as that from the original protein.

Thus while the work described here provides a new and fairly direct demonstration of the presence of glutamine residues in gliadin, it only suggests a minimum figure for the amount of these groups present, and gives no indication of how the greater part of the ammonia set free in protein hydrolysis is bound, or of the mode of linkage of the glutamine residues.

Experimental

N-*Acetylglutamine*

Glutamine, prepared from sugar-beet pulp, was acetylated with NaOH and acetyl chloride in ether [Karrer *et al.* 1926]. It was subsequently found more convenient to use NaOH and acetic anhydride, according to the procedure of du Vigneaud & Meyer [1932] for acetylation of amino-acids. After acetylation, H_2SO_4 equivalent to the NaOH used was added, and the mixture was evaporated to dryness *in vacuo*. Extraction with alcohol and crystallization of the product followed the directions of Karrer. Yield 1·45 g. (by either method) from 3 g. of glutamine. The product melted at 199° and had $[\alpha]_D^{22°}$ −12·25° (water, $l=2$, $c=2·5$). Karrer gives M.P. 199°; $[\alpha]_D^{21°}$ −12·5° (water, $l=1$, $c=1·8$).

Hofmann degradation of N-*acetylglutamine*

It was found that the use of KOH was inconvenient, since potassium forms a sparingly soluble phosphotungstate, and is thus precipitated with the diaminobutyric acid. This difficulty was overcome by using NaOH throughout. Otherwise the conditions given by Kanewskaja [1936] were observed.

Bromine (0·85 g.) was dissolved slowly in a solution of NaOH (0·95 g.) in water (13·3 ml.) at −5°. *N*-acetylglutamine (1 g.) was dissolved in this, and the mixture immediately heated to 80°. After 3–5 min. no iodine was liberated on treating a drop of the mixture with acidified KI solution. The mixture was then treated with 25 ml. 6*N* H_2SO_4, and refluxed until the amino-N (Van Slyke) had reached a constant value (4 hr.). The mixture was then cooled, and diluted to 120 ml. A solution of 20 g. of phosphotungstic acid (B.D.H.) in 60 ml. water was stirred in, and the mixture was kept for 2 hr. at 0° with occasional stirring. The precipitate was filtered off, and decomposed in the usual way with hot aqueous baryta. The filtrate from the decomposition was distilled *in vacuo* for a few minutes to remove ammonia, and amino-N was determined on an aliquot;

55 % of the original *N*-acetylglutamine-N was present in the solution as amino-N. The solution was then made acid to thymol blue with H_2SO_4, filtered through kieselguhr, and the filtrate was concentrated *in vacuo* to a thin syrup, which was treated with a strong solution of flavianic acid. After some hours at 0° the resulting yellow needles were filtered off, and washed with water. Yield 1·95 g. (50 %) l(+)-*αγ-diaminobutyric acid diflavianate*. The product could readily be recrystallized from hot water. M.P. (decomp.) 239°. (Found: C, 38·0; H, 3·15; N (Dumas), 11·19; S, 8·26 %. $C_4H_{10}O_2N_2.(C_{10}H_6O_8N_2S)_2$ requires C, 38·6; H, 2·95; N, 11·26; S, 8·57 %. Found: NH_2-N (Van Slyke, 30 min. shaking), 3·73; calc. 3·75 %.)

The compound yielded 96 % of its amino-N in the Van Slyke apparatus in 6 min. at 18°, and 100 % in 30 min. It had a solubility at 19° of 8·4 mg./ml. in water, 6·2 mg./ml. in 5 % flavianic acid, and 5·9 mg./ml. in *N*/10 H_2SO_4. For comparison with the solubilities and properties of the flavianates of other organic bases, including ornithine and lysine, cf. Kossel & Gross [1924].

It was found unnecessary in preparing diaminobutyric acid from glutamine to work up the intermediate *N*-acetylglutamine. In the procedure finally adopted the mixture from the acetylation of glutamine with acetic anhydride and NaOH was treated directly with alkaline hypobromite, and the resulting diaminobutyric acid isolated as the diflavianate in the manner described above. Overall yield 40–50 %.

Salts of l(+)-*αγ-diaminobutyric acid with oxalic acid*. Karrer *et al.* [1926] characterized *l*(+)-αγ-diaminobutyric acid by means of a salt with oxalic acid, which they described as melting with decomposition at 205° and having the composition of 1 mol. base: 0·5 mol. oxalic acid, without water of crystallization. Fischer [1901] characterized the oxalate of *dl*-αγ-diaminobutyric acid as decomposing at 219° and having the composition 1 mol. base: 0·5 mol. oxalic acid: 1 mol. water.

In the present work it was found impossible to obtain a salt agreeing in properties with that described by Karrer. In all, three different oxalates were obtained; the salt which resulted depended on the amount of oxalic acid added to the solution of the base. The following salts were prepared and characterized:

Compound	Mol. diaminobutyric acid	Mol. oxalic	Mol. water
A	1	0·5	1·5
B	1	1	0
C	1	1·5	0

Of these C is the least soluble in cold water and the most convenient to prepare, as an excess of oxalic acid may be added without fear of contamination of the product by higher salts.

The diflavianate was dissolved in hot water, and treated with excess baryta (alkaline to thymolphthalein). The precipitate of barium flavianate was filtered off, and washed thoroughly with *N*/3 $Ba(OH)_2$. The pale yellow filtrate was acidified to thymol blue with H_2SO_4, stirred with a little charcoal and filtered. Sulphate was removed exactly from the colourless filtrate with baryta, and an amino-N determination on the filtrate from $BaSO_4$ showed that 85 % of the original diaminobutyric acid was present in the solution. 0·5 mol. of oxalic acid per mol. of diaminobutyric acid was then added, and the mixture was concentrated until crystallization occurred. This gave crystals of oxalate A, decomp. 211°. If the diaminobutyric acid was crystallized with 1 mol. of oxalic acid, oxalate B was obtained, decomp. 206°. Compound C could be prepared by

crystallizing from water in the presence of an excess of oxalic acid. Decomp. 177°.

On one occasion, in an attempt to prepare oxalate B, rather more than 1 mol. of oxalic acid was added, and the resulting crystalline product decomposed at 195°. On recrystallization the decomposition point fell to 187°, at which value it remained constant on further recrystallization. This product contained on analysis 1 mol. diaminobutyric acid to 1·25 mol. oxalic acid, but appeared on casual examination with a polarizing microscope to consist of two types of crystal. Debye-Scherrer X-ray powder photographs of this product, and of compounds A, B and C were kindly taken by Mr H. Lipson of the Cavendish Laboratory, Cambridge. Cobalt $K\alpha$ radiation was used, and examination of the photographs showed that compounds A, B and C gave individually characteristic series of lines, while the product in question gave the patterns of B and C superimposed, and must therefore be regarded as a mechanical mixture of these.

Compounds A, B and C could each be recrystallized from water without change in properties. Mixtures of A with B and of B with C had decomposition points intermediate between those of the two components, and less sharply defined.

Table I shows the properties and analyses of the three compounds, and of the oxalate described by Karrer.

Table I. *Oxalates of* l(+)-*αγ-diaminobutyric acid*

Compound	Decomp. °C.	$[\alpha]_D$ (water)	C % Found	C % Calc.	H % Found	H % Calc.	N % Kjeldahl found	% Van Slyke found	N % calc.
A	211	+5·7° +6·0° (c=1·5)	— —	— —	— —	— —	14·8 15·1	14·8 —	14·75 —
A (anhydr.)	211	—	36·7	36·8	7·07	6·74	16·8 (Dumas)	—	17·2
B	206	—	35·3 —	34·6 —	5·92 —	5·77 —	13·2 13·7	13·9 —	13·48 —
C	177	—	32·7	33·2	5·30	5·14	10·8	11·3	11·1
Karrer's oxalate	205	+7·3° +7·8° (c=1·2)	37·0	—	6·50	—	17·04 (Dumas)	—	—

Compound A on drying over phosphoric oxide at 100° *in vacuo* lost 13·35% of its weight. Calc. for 1·5 H_2O per mol. of base: 14·2%.

Compounds B and C (air-dry) lost no weight under the same conditions. Karrer *et al.* [1926] reported their oxalate as anhydrous.

Isolation of diaminobutyric acid from gliadin after hypobromite treatment

Gliadin was prepared from wheat gluten (B.D.H.) by the method of Nolan & Vickery [1937]. The air-dry material contained 7·5% of moisture, and had N 16·2% (Kjeldahl)—corresponding to N 17·5% in the dry protein.

The method of carrying out the degradation of the protein, and isolation of the resulting base described here is that finally adopted as a routine procedure. The effect of varying some of the conditions is described below.

In a typical experiment 1·5 g. of air-dry gliadin were shaken for 2 hr. with 40 ml. *N* NaOH at room temperature. By this time all solid matter had dissolved. The solution was cooled to −5°, and a hypobromite solution, prepared as above from 80 ml. water, 5·6 g. of NaOH and 5 g. of bromine was added. The mixture was then heated rapidly to 80°. After 3 min. practically all the bromine had disappeared (acid KI test), and the almost colourless solution was acidified with 220 ml. HCl (sp. gr. 1·16) and refluxed for 20–24 hr. At the end of this time

the solution was evaporated *in vacuo* until a large quantity of salt had separated. This was filtered off, washed with strong HCl, and the combined filtrate and washings were further concentrated to dryness. The residue was dissolved in 70 ml. water, 4 ml. H_2SO_4 were added, and a solution of 11 g. of phosphotungstic acid in 35 ml. water was stirred in. The rest of the isolation was carried out in the manner already described. The amino-N present in the solution from the decomposition of the phosphotungstic acid precipitate after removal of ammonia was 8·5 % of the total N of the protein used, whereas the corresponding figure for a hydrolysate of untreated gliadin was 4·5 %. On acidifying the solution with H_2SO_4, filtering off $BaSO_4$, and carrying out a silver-baryta precipitation [Kossel, 1928], 60 % of the amino-N from the phosphotungstic acid precipitate derived from untreated gliadin was found to be precipitated, whereas the same procedure, using the treated gliadin, precipitated only 15 % of the amino-N. An experiment with a solution of diaminobutyric acid oxalate showed that none of the base was precipitated by the silver-baryta procedure.

It was found that the silver-baryta precipitation could be satisfactorily omitted, and the diaminobutyric acid isolated by treatment with excess of flavianic acid in the presence of free H_2SO_4 at a volume of 20 ml. After 12 hr. at 0° crystallization was complete; the product on drying decomposed at 237°. Yield 250–320 mg. On recrystallization, the decomposition point was 239°, not depressed on admixture with the diflavianate derived from glutamine. (Found: C, 38·2; H, 3·38; N, 11·3; S, 8·39; amino-N (½ hr. Van Slyke) 3·70 %. Calc. for $C_4H_{10}N_2O_2.(C_{10}H_6N_2O_8S)_2$: C, 38·6; H, 2·95; N, 11·3; S, 8·57; amino-N, 3·75 %.)

A rough estimate of the effect on the yield of varying the conditions of degradation was obtained from the amino-N figure of the solution from the decomposition of the phosphotungstic acid precipitate after removal of ammonia. Table II summarizes the results.

Table II

Temp. of treatment 0° C.	Time of heating min.	Vol. hypobromite solution added (% of amount used above)	Amino-N (as % of original total N)
80	5	20	4·7
80	5	65	7·0
80	3	100	8·5
80	5	130	6·0
80	5	160	4·8
80	5	200	4·0
65	12	160	4·5
50	12	160	5·0

The yield from the degradation carried out under optimum conditions as already described, using 1·5 g. of air-dry gliadin, was corrected for losses in isolation of the base, as follows: *l*(+)-$\alpha\gamma$-diaminobutyric acid oxalate corresponding to 593 mg. of the diflavianate was dissolved with 6·5 g. of NaBr and 6 g. of NaCl in 350 ml. 20 % HCl, and refluxed for 24 hr. The isolation of the base was then carried out exactly as described above, 416 mg. of diflavianate being obtained. Thus the loss in isolation corresponds to about 180 mg. of diflavianate. Applying this correction, the diaminobutyric acid (expressed as free base) is found to be 68–79 mg. from 1·5 g. of air-dry gliadin. (1·39 g. of dry protein.) Expressed as glutamic acid, this is equivalent to 6·1–7·1 % of the dry protein, or, employing the figure of Jones & Wilson [1928] for the glutamic acid content of gliadin as 43 %, 14–16·5 % of the glutamic acid residues present.

Isolation of diaminobutyric acid from an acetylated peptic digest

The same procedure was carried out on an acetylated peptic digest. 1·5 g. of air-dry gliadin were suspended in 54 ml. *N*/20 HCl, and a solution of 80 mg. of a commercial peptic preparation ("Glanoid") in 8 ml. *N*/20 HCl was added, with toluene as antiseptic. The solution had become homogeneous after 2 hr. incubation at 37°. Incubation was continued for 5 days, after which the digest was neutralized with NaOH to phenol red and concentrated *in vacuo* to about 10 ml. It was acetylated with acetic anhydride and NaOH at 0°, using in all 1·7 ml. of acetic anhydride and 20 ml. of 2*N* NaOH. The resulting mixture was then treated with alkaline hypobromite, and after acid hydrolysis the isolation was carried out exactly as described above. Yield of diflavianate was 270 mg., i.e. much the same as that obtained from direct hypobromite treatment of the protein.

When peptic digestion was followed by tryptic digestion and acetylation, the yield of diflavianate on degradation of the resulting mixture was about 50 % of that obtained above.

Recovery of added N-*acetylglutamine*

When 0·10 g. of *N*-acetylglutamine was added to the alkaline solution of 1·5 g. of air-dry gliadin immediately before treatment with hypobromite, 470 mg. of diflavianate were isolated. This is 150 mg. in excess of the best yield obtained from the same amount of protein by itself, and represents a yield of 38 % from the added *N*-acetylglutamine. In another experiment only 10 % recovery was obtained. When it is considered that the yield from added *N*-acetylglutamine is not subject to correction for loss in the course of isolation, it compares unfavourably with the yield obtained by the direct degradation of *N*-acetylglutamine.

C, H, S and N-Dumas determinations were carried out by Dr A. Schoeller, Berlin-Schmargendorf, and Dr G. Weiler, Oxford.

Summary

1. *N*-Acetylglutamine was subjected to the Hofmann degradation with alkaline hypobromite; a 50 % yield of *l*(+)-$\alpha\gamma$-diaminobutyric acid was obtained.

2. A method for the isolation of this base by successive precipitation with phosphotungstic and flavianic acids has been developed.

3. The salts of the base with flavianic and oxalic acids have been characterized.

4. After treatment of wheat gliadin with alkaline hypobromite, followed by acid hydrolysis, *l*(+)-$\alpha\gamma$-diaminobutyric acid was isolated. The best yields, corrected for minimum losses in isolation, were equivalent to 14–16·5 % of the glutamic acid residues of the protein.

5. It is argued that at least this proportion of the glutamic acid of gliadin exists in the intact protein as glutamine residues.

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