42. THE SEPARATION OF AMINO-ACIDS BY MEANS OF THEIR COPPER SALTS

3. THE HYDROLYSIS OF GLIADIN. AN INVESTIGATION OF THE DICARBOXYLATE FRACTION, INCLUDING THE ISOLATION OF *r*-GLUTAMIC ACID AS AN HYDROLYSIS PRODUCT

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THE study of the hydrolysis of gliadin described in this paper was started during the tenure of an Alexander Brown Coxé Research Fellowship at Yale University during the year 1931-32. It is gradually being completed with a view to obtaining as complete a picture as possible of the disposition of the amino-acids in their separation by means of their copper salts.

In the original paper [Town, 1928] three fractions were obtained by separating the copper salts of the amino-acids into:

1. Those soluble in methyl alcohol.
2. Those soluble in water, but insoluble in methyl alcohol.
3. Those insoluble in water.

In a later paper [Town, 1936] the methyl alcohol-soluble copper salt fraction was further subdivided into (a) a part precipitated by alcoholic CdCl_2 and (b) the filtrate. The examination of the CdCl_2 precipitate, which contains the proline, was fully reported.

Modifications were introduced into this hydrolysis as a result of experience gained in previous hydrolyses. Important technical improvements were as follows.

(a) Copper hydroxide was used in place of the basic copper carbonate formerly employed. This substance is much more finely divided and more reactive, and when the amino-acids are evaporated with copper hydroxide to form their copper salts, the calculated quantity is sufficient, no excess being required.

(b) Earlier work had shown that unless the ammonia is first removed, cuprammonium compounds are formed during copper-salt formation and these interfere greatly with the true solubility of the copper salts of the amino-acids in the solvents employed, namely methyl alcohol and water.

(c) The dicarboxylates also interfered in the examination of the methyl alcohol-soluble copper salts [Town, 1936], for it was then found that 2.06 % of the glutamic acid, which should be present in the water-soluble copper salts, was isolated from the methyl alcohol-soluble copper salt fraction. This interfered with the isolation of proline.

The fact that both ammonia and the dicarboxylic amino-acids interfered during the copper salt separation suggested their prior removal. The dicarboxylates were first precipitated by the usual modification of Foreman's method

[Foreman, 1914], in which baryta takes the place of lime. The filtrate containing alcohol was then concentrated under reduced pressure to remove both the alcohol and the ammonia. The solution left was then freed from any excess baryta and converted into copper salts in the usual manner by evaporating with $\text{Cu}(\text{OH})_2$.

The exhaustive examination of the amino-acids precipitated according to Foreman's method or by the common modification of using baryta in place of lime is of prime importance to protein chemists, for conflicting statements have been issued as to the extent that other amino-acids are precipitated as Ca or Ba salts insoluble in alcohol. The results of the present investigation do not seem to bear out any suggestion that other amino-acids are precipitated by the usual procedure. Thus 96.4 % of the nitrogen has been accounted for and the only amino-acids isolated from the dicarboxylate fraction were glutamic acid, aspartic acid and serine. The nitrogen distribution in the fraction is fully indicated in the scheme on p. 431.

The Ba salts of the dicarboxylic acids after precipitation were decomposed and the solution of the amino-acids, containing 27 % of the total N, concentrated and saturated with HCl gas. Five crops of glutamic acid hydrochloride were isolated, the first four crops being homogeneous and having m.p. above 202°. Details of their properties are given in Table 1. It will be noted that 87.5 % of the glutamic acid was isolated in the first crop. The first four crops yielded 254.5 g. of *l*(+)-glutamic acid hydrochloride in practically pure condition, whereas the 5th crop gave 3.0 g. of a fraction with a very low positive rotation and a low melting point. This was at first thought to be a very impure fraction, until it was found much later on to yield an anhydrous 3:5-dinitrobenzoyl derivative, m.p. 204° (51 % yield), whereas *l*(+)-glutamic acid gives rise to a similar derivative, m.p. 104° (40 % yield), crystallizing with one molecule of water.

A complete investigation of the mother liquor from the 5th crop increases the yield of this amino-acid with the 3:5-dinitrobenzoyl derivative, m.p. 204°, to 1.93 % of the dry protein or 1.06 % of the total N. A chemical investigation described below has shown it also to be a glutamic acid, and evidence is adduced which would lead to the belief that it is racemic glutamic acid (as distinct from the *dl* mixture), and that it is an hydrolysis product and not an artefact. The ratio of racemic to *l*(+)-glutamic acid is 1/19.6.

This isolation of racemized glutamic acid from a normal protein is of great interest in view of the discussion which has recently taken place between Kögl & Erxleben [1939, 1, 2 & 3] and Chibnall *et al.* [1939; 1940], concerning the glutamic acid of normal and malignant tissue proteins. The former authors asserted that the glutamic acid isolated from malignant tissue protein was partially racemized and they suggested that it was a characteristic of such proteins. Chibnall found that tumour proteins contained the bulk of their glutamic acid in the ordinary active form and he thought that any racemization was incidental to the methods employed in the isolation of the glutamic acid and had no special significance with respect to cancer tissue.

The isolation of racemic (as distinct from *dl*) glutamic acid from gliadin shows that a normal protein may contain some of its glutamic acid in the inactive racemic form and it may be a general characteristic of proteins. It would appear not to be a criterion of malignancy in living tissue. However, the suggestion that proteins may contain some portion of their constituent amino-acids in an optically inactive form is, as far as the author is aware, a new conception, and it will be interesting to see if in the future other amino-acids are

shown to exist in the same state. The author contributed to a symposium which recently appeared on the subject of the glutamic acid of proteins [Town, 1940].

The total yield of glutamic acid isolated from the sample of gliadin used is 40.4 % of the dry protein or 22.1 % of the total N; these figures are made up as follows:

	% of dry protein	N isolated total N	
<i>l</i> (+)-glutamic acid	37.81	20.7	
<i>dl</i> -(mixture)glutamic acid	0.69	0.38	1.44
<i>r</i> -(compound)glutamic acid	1.93	1.06	
	40.43	22.14	

It is interesting to note that the M.P. of *r*-glutamic acid hydrochloride is 194°, for divergent values for the M.P. of glutamic acid hydrochloride, are quoted in the literature. The following values for the M.P. of the different varieties of this substance have been found during the course of this work: *l*(+)-glutamic acid hydrochloride, 207°; *dl*-, 213°; *r*-, 194°.

The minute examination of the mother liquor from the precipitation of the glutamic acid hydrochloride was originally undertaken with a view to isolating hydroxyglutamic acid, which has been said to be present in gliadin. The isolation of this acid has proved difficult to many workers and although it was first isolated by Dakin as long ago as [1918] from caseinogen, its occurrence as an hydrolysis product of proteins cannot yet be said to have been substantiated. The mother liquors from the precipitation of glutamic acid hydrochloride were first reprecipitated with baryta and alcohol to ensure that only dicarboxylates were present, and then the free amino-acids were converted into copper salts to separate any aspartic acid as its insoluble copper salt. The soluble copper salts were freed from copper, and the free amino-acids treated with silver sulphate and baryta to precipitate any Ag hydroxyglutamate as recommended by Dakin [1918]. The treatment that the precipitate and filtrate from this Ag precipitation underwent is clearly indicated in Diagram 2 and it can be seen that no separation has been effected, for both precipitate and filtrate were found to contain *r*-glutamic acid. No trace of hydroxyglutamic acid was detected during the course of this work, although special care has been taken to look for it, and it is the author's considered opinion that it is not present in gliadin. It is interesting to note that 1.1 g. of serine have been isolated from the final fractions, and it seems probable that a mixture of say *r*-glutamic acid and serine may have been mistaken for this acid.

Another important point which this investigation has raised is the identity of copper aspartate. The copper aspartate isolated during this analysis appeared to be homogeneous, but on decomposition and fractional crystallization of the free amino-acid, it was shown to be a mixture of aspartic acid and *r*-glutamic acid in approximately equal proportions. Whether this is a general behaviour or something peculiar to this analysis has yet to be decided. The author has examined three laboratory samples of aspartic acid and all of them have been found to contain some proportion of the *r*-glutamic acid (the smallest proportion being approximately 4 %). Unfortunately their previous history is unknown. It might be mentioned that although aspartic acid is said to melt at 270°, it has been found in this laboratory that the only sample of aspartic acid which melted at all also contained *r*-glutamic acid and had M.P. 273° (indefinite). All pure samples of this acid prepared in this laboratory have M.P. >320°, and have not yet been made to melt.

EXPERIMENTAL

Preparation of the gliadin. A brief description is given owing to enquiries as to the method of preparation. 5 kg. of a commercial gluten preparation obtained from 'The Battle Creek Food Co.', Battle Creek, Michigan, were extracted with 25 l. and then with 10 l. of 73 % alcohol, each time being left overnight. The alcohol extract was poured off and the gluten residue wrapped in calico and squeezed in a hydraulic press. The extract was a clear yellow liquid (29 l.) and it was concentrated at 45° under reduced pressure to 1/3 volume. It was poured into ice-cold water and the gliadin precipitated as a thick viscous material which was transferred to a large basin and treated with three changes of dry acetone. The gliadin fell rapidly away to a fine powder and any lumps were passed with acetone through a hand mincing machine. The powder was left with dry acetone for several hours, filtered on a Büchner funnel, and spread on brown paper to dry.

Properties. The preparation is a fine white powder and dissolves in 73 % alcohol to a clear colourless solution. It contains 15.2 % N, 10.22 % moisture and 1.08 % fat (ether-soluble material). The N on a moisture and fat free basis is 17.40 %. The specific rotation using a 7 % solution of the air-dried specimen was -79.5° , which is equivalent to -88.8° for the anhydrous protein.

Hydrolysis

600 g. of the air-dry protein (532 g. anhydrous protein) were boiled under reflux for 36 hr. with 30 % w/v H_2SO_4 . The total N in the hydrolysed protein was 91.6 g. The hydrolysed solution (3200 ml.) was diluted with 5100 ml. water and the humin material filtered off (8.6 g.; N, 0.3 g.). The filtrate was treated with hot aqueous baryta until just acid to Congo red, the BaSO_4 being filtered and washed with acidified boiling water (wt. BaSO_4 , 2214 g.; N, 3.02 g.). The filtrate and washings were concentrated to 2870 ml. (N, 88.0 g.) and 786 g. of baryta in hot water added. To the resulting 5 l. of solution, 15 l. 95 % alcohol were added with constant stirring and the powdery but bulky precipitate of barium dicarboxylate allowed to settle overnight before filtration. The bulk of the supernatant alcohol, which was alkaline to litmus, was siphoned off and concentrated under reduced pressure (bath at 50°) to remove the ammonia before converting into copper salts. The Ba-dicarboxylate precipitate was finally filtered and washed once with 60 % alcohol.

The dicarboxylate precipitate. The precipitate of Ba salts was dissolved in dil. HCl (vol. solution 3 l.) and the Ba removed quantitatively; the BaSO_4 precipitate was washed twice by boiling with 800 ml. 5 % HCl (wt. BaSO_4 , 740 g.; N, 0.86 g.). The amino-acid solution after concentration had volume 2300 ml. (N, 24.61 g.; 26.97 % of total N (91.62 g.)) and the concentration was continued until glutamic acid hydrochloride commenced to crystallize (vol. 1500 ml.). The solution was now cooled in ice and saturated with HCl gas; a large crystalline precipitate formed. It was filtered off, dissolved in 1200 ml. water and reprecipitated by saturation with HCl gas. The precipitate was filtered, and weighed 225 g. (1st crop).

The mother liquors were concentrated to 400 ml. and again saturated with HCl gas, giving a second crop of crystals which on recrystallization weighed 15 g. There was similarly obtained a 3rd crop (wt. 7.5 g.); the solution was now very dark brown and so it was boiled with norite (0.5 g.), filtered, concentrated to 100 ml. and again treated with HCl gas (4th crop, wt. 7.0 g.); on saturating the mother liquor (30 ml.) with HCl a 5th and final crop of hydrochloride was obtained (wt. 3.0 g.). The properties of these five crops of hydrochlorides are shown in Table 1, while the treatment of the mother liquor is described later.

Table 1

Crop	Wt. of hydrochloride g.	M.P. of hydrochloride ° C.	Wt. as free glutamic acid g.	% N	% Cl	Test for NH ₃	[α] _D ($e=4$ in H ₂ O)	Yield	
								N/total N %	Amino-acid Protein %
1	225	207	181	7.55	19.40	—	+ 25.0	18.5	34.0
2	15	203	12.1	7.40	19.45	—	+ 20.8	1.1	2.3
3	7.5	202	6.0	7.43	19.5	—	+ 19.1	0.6	1.1
4	7.0	202	5.6	7.40	19.4	—	+ 24.0	0.6	1.1
5	3.0	175	2.4	7.45	19.3	Just + ve	+ 5.8	0.25	0.5
Total	257.5		207.1					21.05	39.0
								Excluding 5	20.8
									38.5
Properties of glutamic acid hydrochloride:									
Softens		203		7.63	19.34		+ 25.0		
M.P.		207							

Although the information in Table 1 was collected over a long time it has been put together without further explanation, although it should be pointed out that the rotations have only recently been determined.

The yield of glutamic acid expressed in terms of *l*(+)-, *d*(-)- and *dl*-mixture as calculated from the rotations is as follows:

	% of protein			
	I		II	
	<i>l</i> (+)-acid	<i>dl</i> (mixture)-acid	<i>l</i> (+)-acid	<i>d</i> (-)-acid
Crop 1	34.0	0.0	34.0	0.0
Crop 2	1.91	0.39	2.11	0.20
Crop 3	0.84	0.26	0.97	0.13
Crop 4	1.06	0.04	1.08	0.02
	37.81	0.69	38.16	0.35

To this has to be added the amount of glutamic acid isolated from crop 5 and later fractions which is thought to be *r*-glutamic acid, namely 1.93 % of the dry protein. This makes a total yield of 40.4 %.

Examination of mother liquor from the precipitation of glutamic acid hydrochloride

The mother liquor from the 5th crop was evaporated to a syrup and then diluted to one litre; 25 ml. 12 *N* H₂SO₄ were added and the HCl removed by adding Ag₂O to slight excess (test with baryta), excess Ag being removed with H₂S. The precipitates were washed by boiling twice with water (AgCl, wt. 58.6 g.; N, 0.01 g.; Ag₂S, wt. 1.3 g.; N, negligible); the filtrate and washings were concentrated to 710 ml. (N, 5.47 g.). It was decided at this stage to reprecipitate the dicarboxylates, if any, with the group reagent baryta and alcohol: 220 g. Ba(OH)₂ · 8H₂O were added and the combined volume was 800 ml.; 3 vol. 95 % alcohol were added and the granular precipitate allowed to settle, filtered and washed. Weight of precipitate 180 g. (N, 3.34 g.). The filtrate (3660 ml.; N, 2.02 g.) was evaporated under reduced pressure and the N in the aqueous residue determined (vol. 495 ml.; N, 1.46 g.). This indicates that 2.02–1.46, or 0.55 g., N was still present as ammonia and had distilled over. The distillate gave strongly positive tests for NH₃.

The aqueous residue (N, 1.46 g.) was converted into copper salts and mixed with the main bulk of copper salts of the monoamino-acids obtained after the

original precipitation of the dicarboxylates. This action was later regretted, as it would have been interesting to see what amino-acids were precipitated with the dicarboxylates at their first precipitation, but one is in great danger of needlessly multiplying fractions. Before proceeding further, a schematic diagram will show the exact stage reached in the separation (Diagram 1).

Diagram 1

The precipitated Ba dicarboxylates (N, 3.34 g.) were dissolved in one litre of water and freed from Ba (wt. BaSO_4 , 120 g.; N, 0.26 g.). The amino-acids were converted into copper salts by boiling with 25 g. Cu(OH)_2 , filtered from excess Cu(OH)_2 (wt. 12 g.; N, 0.33 g. after boiling three times with water), and left in the ice-chest to cool; a mass of light blue feathery crystals of copper aspartate separated (wt. 12.1 g.; N, 0.61 g.; this represents 1.09 % of the dry protein or 0.66 % of total N; loss in weight on drying is 24.6 %; however, see later.)

The filtrate from the copper aspartate was freed from Cu (wt. CuS, 11 g.; N, 0.016 g.) and the solution concentrated to 1050 ml. (N, 2.04 g.). This solution was now treated as described by Dakin [1918] for the isolation of hydroxy-glutamic acid. It was treated with precipitated AgOH and a little sulphuric acid (just sufficient to keep it acid to Congo red) until excess of Ag was present (brown precipitate with baryta). Baryta water was now added until pH 7 was reached and the white precipitate filtered off; on bringing the filtrate to pH 10 a small black precipitate formed which was also filtered off. The final filtrate had vol. 1430 ml. (N, 1.30 g.) and so the quantity of N precipitated should be 0.74 g., i.e. 36.3 % of the total. All fractions were freed from Ag (Ag₂S from precipitates 27.3 g.; N, 0.01 g.; Ag₂S from filtrate 13 g.; N, 0.006 g.) and the solutions concentrated on the water bath (65°) under reduced pressure.

Ag precipitate. On evaporation, tetrahedral crystals separated rather like glutamic acid, but the solution was evaporated to dryness and extracted by grinding with glacial acetic acid and left overnight. Only a small portion dissolved; the insoluble material being centrifuged off and again treated with acetic acid for 12 hr. Finally it was dissolved in 2 ml. H₂O and saturated with HCl gas (wt. of precipitate 1.7 g.). At first this was thought to be ordinary glutamic acid hydrochloride and was not examined. Later, however, on attempting to prepare the 3:5-dinitrobenzoyl derivative it was found to yield mainly a derivative, M.P. 204°, together with a trace of the normal glutamic acid derivative, M.P. 104°. Yield: 0.64 g. amino-acid hydrochloride gave 0.31 g. derivative, M.P. 204° (26.1 % of theory) and 0.02 g. derivative, M.P. 104° (1.5 %).

The material soluble in acetic acid was treated with methyl alcohol to cause granulation, dried and again treated with acetic acid. A trace (0.16 g.) was insoluble. The solution was concentrated to 30 ml. and poured into one litre of acetone when the material granulated: wt. 2.05 g.; N, 0.19 g.

Filtrate from Ag precipitation. As evaporation proceeded crystals separated in long needles which were identified as tyrosine (wt. 0.15 g.; N, 7.5 %; Millon positive); later a further crop of tetrahedral crystals separated (wt. 1.5 g., M.P. 197°), and finally a 3rd crop, also tetrahedra, containing a few coarse needles (wt. after crystallizing 0.8 g., M.P. 187–188°). Later it was found that both these fractions yielded 3:5-dinitrobenzoyl derivatives of M.P. 204° identical with the material obtained from the Ag precipitate. No further crops of crystals could be obtained and the syrupy residue was treated with glacial acetic acid, when all but 0.3 g. dissolved. The solution was evaporated *in vacuo* over NaOH and re-dissolved in glacial acetic acid, but 0.4 g. was insoluble. This 0.7 g. material was also found to yield a 3:5-dinitrobenzoyl derivative, M.P. 202°. The acetic acid solution was concentrated and granulated by pouring into one litre of acetone. The bulk of the material was insoluble (N 0.68 g.), but the acetone solution was slightly coloured; it was mixed with that from the Ag precipitate and was found to contain 0.24 g. N.

As the precipitation with silver and baryta according to the method of Dakin had apparently effected no separation the fractions were recombined to give (a) material insoluble in acetone containing 0.68 + 0.19 or 0.87 g. N; (b) material soluble in acetone (0.24 g. N). On evaporation both solutions gave syrupy residues and, after many experiments, these were dissolved separately in 50 % methyl alcohol and precipitated by adding a 20 % solution of mercuric acetate in 50 % methyl alcohol.

Acetone-soluble. N precipitated 0.10 g. (41.5 %); this was not examined further as it is probably diketopiperazine material.

Acetone-insoluble. Volume 310 ml. N precipitated 0.66 g. (76 % of total)

The mercury precipitate was decomposed and the solution on evaporation gave a syrup which on stirring suddenly solidified to a waxy solid; this became powdery on treatment with methyl alcohol (wt. 3.58 g.; N, 11.1 %; amino-N 97.5 %, M.P. 165°, softens 140°, no charring). The equiv. wt. by titrating in alcohol solution was 104; it formed a blue copper salt which did not crystallize. A further batch of this crystalline material (wt. 1.4 g.; N, 11.0 %) was obtained by evaporation and treatment with MeOH.

As this material seemed to be a mixture it was decided to mix the fractions and reprecipitate them with mercuric acetate. The N content of mixed crops was 0.54 g., of which 0.46 g. (87 %) was precipitated. The Hg precipitate was decomposed and the amino-acid solution evaporated under reduced pressure. It was difficult to work with, but on adding methyl alcohol till the solution was turbid and keeping, a quantity of hard gritty crystals formed (wt. 0.51 g.; N, 9.73 %; M.P. 188°), crystallizing in rectangular prisms. This was also later found to yield a 3:5-dinitrobenzoyl derivative M.P. 202°. The mother liquor on evaporation and retreatment with methyl alcohol yielded more crystals, also gritty to the touch (wt. 0.46 g.; N, 9.65 %). Its 3:5-dinitrobenzoyl derivative had M.P. 202°.

The residual syrup on long keeping became semi-solid, it was scraped out, filtered, dried for 3 weeks over conc. H_2SO_4 (wt. 1.12 g.); N, 11.8 %; moisture content 6.15 %; N in dry material 12.6 %). It was thought to be serine both from its N content and because it precipitated even in dilute solution with mercuric acetate in 50 % methyl alcohol, a property which the author has found to be characteristic of serine. It also yields a 3:5-dinitrobenzoyl derivative, M.P. 180°, mixed M.P. with a known *l*-serine derivative 181°, softens 179° (serine derivative, M.P. 183°).

The steps taken in the investigation of the water-soluble copper salts from the reprecipitation of the dicarboxylates, containing originally 2.04 g. N, are indicated schematically below (Diagram 2).

Examination of copper aspartate. This fraction which, from an examination of the feathery crystals of the copper salt, appeared to be homogeneous, was examined because the water content, 24.6 %, corresponded with 3.25 mol. of water of crystallization, whereas the figure quoted in the literature is 4.5 mol. The copper aspartate left, anhydrous wt. 7.12 g., was dissolved in 60 ml. *N* HCl, freed from copper and the solution evaporated and crystallized. By a complex process of crystallization from water and from alcohol, the free amino-acid was found to be a mixture. There was isolated 2.25 g. aspartic acid; M.P. > 320°; C, 35.78; H, 5.00, N, 10.39 %: theory C, 36.08, H, 5.26, N, 10.53 %, and also 2.30 g. of an amino-acid, M.P. 194°, softening at 191°, which gave on analysis figures corresponding to glutamic acid; found, C, 40.2, H, 6.56, N, 9.76 %: theory C, 40.7, H, 6.90, N, 9.52 %: however, on treatment with 3:5-dinitrobenzoyl chloride it gave a derivative, M.P. 202°, softening at 200°, whereas natural glutamic acid yields a derivative M.P. 104°. As mentioned later in this paper this amino-acid is thought to be racemic glutamic acid, and what was thought to be copper aspartate is apparently a mixture or a double salt of copper aspartate and copper *r*-glutamate approximately in the ratio 1:1. This of course may be a phenomenon peculiar to this hydrolysis, but it is of great importance to ensure the purity of copper aspartate as aspartic acid is so frequently estimated as its copper salt.

Examination of the crops of glutamic acid hydrochloride. The details of the routine examination of these five crops are given in Table 1, and need no comment except to point out the low M.P. of crop 5 and to note its low rotation.

Diagram 2

All the fractions marked with an asterisk were found to give a 3:5-dinitrobenzoyl derivative m.p. 204°. This is the material which is considered to be *r*-glutamic acid. See p. 426 for full description.

Attempts were made to prepare the *p*-nitrophenylosazone of malic semi-aldehyde according to the method of Dakin [1918] from all these five crops in order to see if there was any β -hydroxyglutamic acid in the fractions, for its hydrochloride is known to be insoluble in HCl: 0.15 g. of each crop was used in the experiment and in each case crops 1, 2, 3 and 4, yielded no crystals from nitrobenzene. Crop 5 yielded 0.5 mg. of a dark red crystalline solid, m.p. 299° (uncorr.). This corresponds to a yield of 0.17 %, assuming crop 5 to be hydroxyglutamic acid for calculation purposes. The substance gave only the faintest blue colour with alcoholic NaOH, whereas *p*-nitrophenylosazones are said to give an intense blue colour with this reagent. These results were not very encouraging, and the fractions were put on one side.

Later [Town, 1941], it was found that glutamic acid yielded a well-characterized 3:5-dinitrobenzoyl derivative and so this derivative was prepared from each crop (see Table 1); 0.80 g. of material was used in each case equivalent to 1 g. acid chloride; only 0.4 g. of crop 5 was used.

	Wt. of deriv. g.	M.P. °	Yield %
Crop 1	0.62	104	40
Crop 2	0.62	104	40
Crop 3	0.61	104	39.4
Crop 4	0.59	104	38.1
Crop 5	0.40	203	51.6 calc. as glutamic acid deriv.

It can be seen from the m.p. and yield that crops 1-4 are undoubtedly homogeneous and consist of glutamic acid hydrochloride. Crop 5 is, however, quite different for it yields a derivative of much higher m.p. and also in a 25 % greater yield from only half the starting material. Careful attempts, not detailed here, have been made by fractionation to see if other substances were present, but no other 3:5-dinitrobenzoyl derivative than the one with m.p. 203° was isolated from this fraction.

After this, the same derivative was prepared from all the fractions which had been obtained in the attempt to isolate hydroxyglutamic acid from the residues from the precipitation of the glutamic acid as hydrochloride. It can be seen below that most of them consisted almost completely of the amino-acid yielding the 3:5-dinitrobenzoyl derivative with m.p. 203°. A similar amino-acid was also isolated by fractional crystallization of the aspartic acid obtained from the copper aspartate.

*Examination of the amino-acid yielding a 3:5-dinitrobenzoyl
derivative with m.p. 203-204°*

It is first of all important to study the disposition of this acid in the scheme of separation employed.

It occurs in crop 5 of the glutamic acid separation (Table 1); therefore its hydrochloride is sparingly soluble in strong HCl. It is present in the copper aspartate fraction, and therefore it either forms an insoluble double salt with copper aspartate or else its copper salt is itself sparingly soluble. It occurs both in the silver precipitate and filtrate on treatment with silver and baryta, but it is present to a greater extent in the filtrate; precipitation is thus only partial. The amino-acid is also partially soluble in glacial acetic acid.

The weights of material obtained from the various sources (see diagram 2) and also the yields of the 3:5-dinitrobenzoyl derivative obtained from the various fractions in which it occurs are indicated below. It is interesting to note

that the yield of derivative is usually of the order 50–60 %, compared with one of 40 % in the case of *l*(+)-glutamic acid (see p. 426).

	Free amino-acid g.	Yield of deriv. %	
As hydrochloride 3.0 g. (crop 5)	2.4	51.6	
From Ag precipitate (diagram 2)	1.7	26	
From Ag filtrate—2nd crop	1.5	24	
From Ag filtrate (Table 3)—3rd crop	1.1	69	
0.79 g. insol. in glac. HAc	0.8	62	
0.51 g. from precipitation with HgAc ₂	0.5	58	
0.46 g. with HgAc ₂	0.46	17	neglected
	8.0		

Also the copper aspartate (q.v.) yielded 2.3 g. of the same amino-acid giving a derivative melting at 202°. There have thus been isolated 10.3 g. of the material corresponding to a yield of 1.93 % of the dry protein or 1.07 % of the total N.

The hydrochloride of the amino-acid. Crop 5 of the hydrochlorides (Table 1) was dissolved in 17 ml. H₂O and saturated with HCl gas; on cooling and rubbing, it crystallized in fine needles, m.p. 194°, wt. 0.815 g. (wt. of starting material 1.570 g.). The mother liquor on evaporation to $\frac{1}{4}$ bulk was brown in colour and on cooling yielded a second fraction, wt. 0.29 g.; m.p. 194°. The two fractions were mixed and recrystallized and the derivative still had m.p. 194°. (Found: C, 32.87; H, 5.30; N, 7.53; Cl, 19.26 %. C₅H₉O₄N.HCl requires C, 32.7; H, 5.45; N, 7.64; Cl, 19.35 %.) The amino-acid hydrochloride thus corresponds in composition with glutamic acid hydrochloride. A m.p. of 194° is among those quoted in the literature for glutamic acid hydrochloride, but it was not then appreciated that it could be the racemic variety.

The 3:5-dinitrobenzoyl derivative. This derivative was prepared in a similar manner to that of glutamic acid [Town, 1941]. It crystallizes in very thin hexagonal plates, m.p. 204°, which have a great tendency to overlay one another so that it is apt to crystallize in a large mass with only the ends of the crystals apparent. The crystal plates are so thin that good definition is difficult to obtain in photography. Photographs of the crystals have been obtained and copies can be sent to interested readers. The ordinary glutamic acid derivative, m.p. 104°, crystallizes in masses of very long fine needles. A 0.8 % solution in glacial acetic acid of the derivative melting at 204° gave no detectable rotation.

This derivative has no water of crystallization (the ordinary glutamic acid derivative crystallizes with 1 H₂O). (Found: C, 42.99; H, 3.15; N, 12.10 %; a substance of formula C₁₂H₁₁N₃O₉ has C, 42.4; H, 3.23; N, 12.30 %; which would correspond with the derivative from an acid of the formula C₅H₉O₄N.)

The free amino-acid. The residues of the free amino-acid, after the preparation of the 3:5-dinitrobenzoyl derivatives from all of them, were mixed (wt. 1.633 g.) and crystallized from 10 ml. H₂O; crystals readily separated on cooling, wt. 1.334 g., m.p. 203.5° softening slightly at 200.5°. The mother liquor on evaporation to 3 ml. deposited more crystals; wt. 0.042 g., m.p. 202.5°. The crystals were tetrahedral in shape, very hard to the touch, and practically insoluble in cold water. (Found: C, 41.02; H, 6.06; N, 9.38 %. C₅H₉O₄N requires C, 40.7; H, 6.13; N, 9.52 %.)

The free acid thus also corresponds in composition with glutamic acid.

It was also determined that the ratio of amino-N/total N was 1/1.

Racemization of known glutamic acid. It was suggested that the material might be *dl*-glutamic acid and so 15 g. pure *l*(+)-glutamic acid were racemized

by boiling in *N* NaOH and the rotation determined at 4 hr. intervals. Racemization was complete after 130 hr. boiling. The solution was decolorized with animal charcoal and after evaporation was extracted three times with 80 % alcohol. This extract in aqueous solution was saturated with HCl gas, and there were obtained 4.2 g. of pure *dl*-glutamic acid hydrochloride, m.p. 213° (sharp).

This yielded a 3:5-dinitrobenzoyl derivative (49 % yield), m.p. 104°—this seems to be the point at which the material dissolves in its own water of crystallization. The anhydrous derivative has m.p. 182°, softening at 179°. (Found: 5.05, 5.10 % H₂O corresponding to 1 H₂O per mol., similar to the glutamic acid derivative.) Thus the amino-acid isolated is not *dl*-glutamic acid.

The 3:5-dinitrobenzoyl derivative was also prepared from a sample of *d*(-)-glutamic acid kindly supplied by Prof. A. C. Chibnall; 0.1 g. of the acid gave a 62 % yield of the derivative which had m.p. 105°, mixed m.p. with known 104° derivative, 104°, softened at 101°. Loss in weight at 110° 5.12 %.

The possibility also existed that the amino-acid isolated might be methionine, which with a formula C₆H₁₁SO₂N would give very similar analytical figures, but tests for S by Na fusion were negative. Before it was considered that the acid might be *r*-glutamic acid, it was thought probable that it might be either methylaspartic acid or the lactone of hydroxyglutamic acid and a decision could only be made by oxidation experiments.

Oxidation of the 3:5-dinitrobenzoyl derivative. It was decided to use this derivative in order to protect the amino group. 169 mg. of the derivative were dissolved in 5 ml. glacial acetic acid and the boiling solution titrated with 33.2 ml. *N*/10 KMnO₄ over the space of 5 min., equivalent to 2 atoms oxygen per mol. of derivative. The MnO₂ was filtered off and the solution evaporated on the water bath; solid separated when the volume was 3 ml.; wt. 47.5 mg., m.p. 164°, softened 159°. This, on crystallizing from 5 ml. boiling water, gave 21.4 mg. derivative, m.p. 182.5°, softening at 182°, crystallizing in rosettes of fine needles. (Found: C, 40.95; H, 2.57; N, 12.43%. C₁₁H₉N₃O₉ or C₄H₆O₄N·C₇H₃N₂O₅ would require C, 40.45; H, 2.75; N, 12.43 %.) This derivative was not natural aspartic acid 3:5-dinitrobenzoyl derivative, however, in spite of its similar m.p., because a mixed m.p. with the derivative (m.p. 184.4°) gave m.p. 110° softening at 90°. Work is continuing on its identity.

Titration of the acid with alcoholic NaOH. 60 mg. of the acid in 5 ml. H₂O and 50 ml. ethyl alcohol were titrated with *N*/10 NaOH using thymolphthalein as indicator. As a check 60 mg. ordinary glutamic acid were treated similarly. Both solutions were back-titrated with *N*/10 HCl using methyl red as indicator. In both cases the ratio of COOH/NH₂ groups was 1.88/1 indicating that the acid is a dibasic monoamino-acid. The mol. wt. of the acid is 147.8 (glutamic acid 147).

The silver salt. The solution from the above titration was treated with an exact equivalent of *N*/10 AgNO₃ to free from Cl⁻ (wt. AgCl 14.5 mg.; should be 67.5 mg. thus some AgCl is retained in solution). The solution was decolorized by boiling with 0.25 g. charcoal and evaporated to a syrup; 150 mg. AgNO₃ in 2 ml. H₂O were added and the yellowish-white silver salt filtered off; wt. 120.2 mg. (theory is 147 mg. silver salt). This silver salt went black within an hour or so, and contained 60.1 % Ag. It was dissolved (112.3 mg.) in 5 ml. *N*/10 HNO₃, filtered from a trace of black residue (3.5 mg.) and brought to pH 7-8 (bromothymol blue) by adding 4 ml. *N*/10 NaOH; a whitish precipitate formed (46.7 mg.) which contained 8.49 % H₂O and 58.5 % Ag in the anhydrous salt. This salt also blackened readily on exposure to light. An acid of mol. wt. 147 would give a salt with 59.8 % Ag.

The copper salt. 28.6 mg. of acid in 5 ml. H_2O were boiled with 24 mg. $Cu(OH)_2$ (theory 19.3 mg.) and filtered; the deep blue solution on evaporation gave a blue glass, readily soluble in water, and readily precipitated by adding an equal volume of methyl alcohol as a bulky light blue amorphous powder. % Cu, 19.7. Anhydrous copper glutamate contains 30.3 % Cu, if Cu/N is 1/1.

Electrometric titration. The author's thanks are due to Prof. C. R. Harington for kindly arranging for an electrometric titration to be carried out on the amino-acid, and assuming the mol. wt. to be 147, the following pK values were determined. The figures for glutamic and aspartic acids are given for comparison.

	Amino-acid	Glutamic acid	Aspartic acid
pK_1	2.25	2.19	2.09
pK_2	4.17	4.28	3.87
pK_3	9.44	9.66	9.82

The figures afford strong evidence that the acid isolated is identical with glutamic acid.

Reduction. Attempts were made at reduction of the amino-acid using hydrogen in the presence of Pd adsorbed on charcoal as a catalyst to eliminate all possibility of it being the lactone of hydroxyglutamic acid. No evidence of the absorption of hydrogen was observed.

Oxidation. As the amino-acid thus undoubtedly appears to be a glutamic acid, it was decided to oxidise it with chloramine-T or other source of hypochlorous acid to β -cyanopropionic acid and thence by hydrolysis to succinic acid. Free hypochlorous acid was prepared by distilling a solution of Cl_2 water after treating it with mercuric oxide. On the basis that 1 g.-mol. of glutamic acid requires 2 mol. of $HOCl$ (i.e. 4 equiv.) for oxidation to cyanopropionic acid, the exact equivalent of $HOCl$ was added. 14.7 mg. of acid in 10 ml. solution were treated with 1.31 ml. $HOCl$ (0.307 N) at 40° . After 30 min. the solution still gave a blue colour with starch-iodide paper, which became negative after warming for 2 min. The solution was evaporated on a water bath; 5 ml. 5 N HCl were added and the solution evaporated to dryness. On keeping crystals formed; these were extracted with ether and yielded 5.7 mg. succinic acid, m.p. 181° , mixed m.p. with authentic succinic acid, $182-184^\circ$. A control experiment with 14.7 mg. of $l(+)$ -glutamic acid yielded 6 mg. of succinic acid, m.p. $182-184^\circ$.

pH of aqueous solution of the acid. The pH of $M/100$ solutions (14.7 mg. in 10 ml. solution) of known and new glutamic acids were determined electrometrically and found to be respectively 3.35 and 3.31. This also tends to confirm the suggestion that the unknown acid is a glutamic acid.

Hydrolysis of 3:5-dinitrobenzoyl derivative of the acid. 210 mg. of the derivative were heated in a sealed tube at 120° with 5 ml. conc. HCl for 1 hr.; after cooling, the 3:5-dinitrobenzoic acid was filtered off: wt. 92 mg., 70 % theory. The solution was evaporated to give the free amino-acid hydrochloride (wt. 133 mg.), and this was dissolved in 9 % HCl and made up to 3 ml. The rotation was determined and the concentration of amino-acid by Kjeldahl analysis. Concentration, 95.6 mg. per 3 ml. solution; rotation, -0.15° ; hence $[\alpha]_D = -2.36^\circ$. Some doubt is felt about this rotation as the solution was slightly turbid and in the 2 dm. tube used it was very difficult to read; however, the value -0.15° is the mean of 24 readings. The value may well have been zero when compared with the value obtained below with known glutamic acid.

A similar experiment was carried out on the 3:5-dinitrobenzoyl derivative of known $l(+)$ -glutamic acid. 220 mg. hydrolysed yielded 114 mg. 3:5-dinitrobenzoic acid (78 % theory) and the amino-acid was treated as above. Concen-

tration of solution 76.7 mg. per 3 ml.; rotation $+0.64^\circ$; and $[\alpha]_D = +12.5^\circ$. If this specific rotation is accurate and this solution was also slightly turbid, then it would appear that in the benzylation process the *l*(+)-glutamic acid was racemized to the extent of 60 %; i.e. 30 % of the material was converted into its opposite enantiomorph, which however existed as the *dl* mixture and not as the racemic compound, for the 3:5-dinitrobenzoyl derivative had m.p. 104° , whereas the new amino-acid isolated yielded a derivative m.p. 204° and this is thought to be the racemic compound.

Re-benzylation of the amino-acids obtained above. After the rotations of the free amino-acid hydrochlorides had been determined, the HCl was removed by evaporation and the residues treated with 0.15 g. of 3:5-dinitrobenzoyl chloride and NaOH in the usual manner.

The amino-acid from the derivative which had m.p. 204° gave a product which, after benzene extraction and crystallization of the benzene-insoluble portion from water, yielded 50 mg. of the 3:5-dinitrobenzoyl derivative and this still had m.p. 204° . The yield was 42.6 % calculated on the 85 mg. of acid which were left for this purpose. This corresponds with yields of the order of 50 % which were obtained using 10 times the quantity of starting material and would appear to indicate that no change has been caused by the repeated benzylation.

The amino-acid from the derivative with m.p. 104° , which is that obtained from *l*(+), *d*(-) and *dl*-glutamic acids, yielded by this same treatment 8 mg. of m.p. 204° derivative (a yield of 4.5 %); a mixed m.p. with known 204° derivative gave m.p. 201° , shrinking at 198° . Nothing more could be crystallized from the mother liquor, although careful attempts were made to increase the yield.

This experiment confirms the fact that the amino-acid which yields a 3:5-dinitrobenzoyl derivative m.p. 204° is a glutamic acid and also that it is the racemic compound, for all the other possibilities have been tried and found to yield the 104° m.p. derivative.

Although the *l*(+)-glutamic acid does, after two benzylation, yield small quantities of the derivative with m.p. 204° , it is important to note the small yield in this case, only 4.5 %, compared with a yield of 42.6 % in the case of the amino-acid which originally gave the derivative with m.p. 204° . It can thus be seen that slight conversion of ordinary glutamic acid into the racemic compound does take place after repeated benzylation, but compared with the yields obtained in the course of this analysis (from 50 to 60 %) after only *one* benzylation it is negligible.

All the known experimental facts can be reconciled if it is presumed that the original amino-acid giving a 3:5-dinitrobenzoyl derivative m.p. 204° was racemic glutamic acid and owing to the great difficulty experienced in inducing ordinary *l*(+)-glutamic acid and also known *dl*-glutamic acid to form such a derivative, it is suggested that the *r*-glutamic acid is a hydrolysis product and not an artefact. It is hoped, when opportunity offers, to carry out investigations to determine the equilibrium conditions between *dl*- and *r*-glutamic acids.

Conclusion

The dicarboxylate fraction of gliadin has been exhaustively examined and the partition of the nitrogen in the various fractions is shown in the following balance sheet. Corrections have not been made for the nitrogen used in Kjeldahl determinations, as the nitrogen otherwise unaccounted for is considerably greater.

Nitrogen distribution in the dicarboxylate fraction of gliadin

	Isolated	Original N content
<i>l</i> (+)-glutamic acid	19.0 g.	24.6 g.
<i>dl</i> (mixture)-glutamic	0.35 g.	
<i>r</i> (compound)-glutamic	0.97 g.	
•		20.32 g.
Non-dicarboxylate N after reprecipitation of mother liquors with baryta and alcohol		1.46 g.
Ammonia present		0.55 g.
Aspartic acid (2.25 g.)		0.24 g.
Serine		0.13 g.
Tyrosine		0.01 g.
		22.71 g.
	Losses	
On AgCl	0.01 g.	
On BaSO ₄ (2nd precipitation)	0.26 g.	
On excess Cu(OH) ₂	0.33 g.	
On CuS	0.03 g.	
On Ag ₂ S	0.02 g.	
Soluble in acetone	0.24 g.	
Hg(Ac) ₂ filtrate	0.08 g.	
Loss in crystallization of aspartic acid	0.04 g.	
		1.01 g.
		23.7(2)
Unaccounted for		0.89 g.
		24.6(1) g.

Thus 23.7 g. N out of a total of 24.6 g. are accounted for, or 96.4 %, although just over 4 % represents unavoidable losses on precipitates. The only amino-acid present besides glutamic and aspartic acids, except for 1.46 g. N which did not precipitate with baryta and alcohol on the second treatment with this reagent (and which was mixed with the main bulk of copper salts), is a small quantity of serine. This is a very soluble amino-acid and its presence no doubt had a very profound effect on the crystallization of the final fractions. The presence of serine always tends to prevent the crystallization of other amino-acids.

SUMMARY

An investigation into the dicarboxylate fraction of gliadin, obtained by precipitating the dicarboxylates from the hydrolysed protein by means of baryta and alcohol, has been completed.

The yield of glutamic acid amounts to 40.4(3) % of the dry protein, or to 20.7 % of the total N.

Besides the ordinary *l*(+)-glutamic acid, there was isolated a new variety of glutamic acid which is believed to be racemic glutamic acid. This is thought to be a definite hydrolysis product and not an artefact. The ratio of racemic to *l*(+)-glutamic acid is 1/20.

The aspartic acid was isolated as a well-crystallized copper salt, but this was found to be a mixture, and to approximate to a double salt of copper *r*-glutamate and copper aspartate. This result may be peculiar to this analysis, but it indicates the necessity of ensuring the purity of copper aspartate in this method of estimation.

The yield of pure aspartic acid was 0.43 % of the dry protein, or 0.26 % of the total N.

The only other amino-acid present was serine, which was isolated to the extent of 0.18 % of the dry protein, or 0.14 % of the total N. It was identified

by its ready precipitation with mercuric acetate in 50 % methyl alcohol, and by its 3:5-dinitrobenzoyl derivative. This amino-acid no doubt accounted for the intractability of the mother liquors from the glutamic acid.

There seems no ground for the belief that baryta and alcohol, at least after a second precipitation with this reagent, bring down considerable quantities of other amino-acids.

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