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

II. AN INVESTIGATION OF THE METHYL ALCOHOL- SOLUBLE COPPER SALT FRACTION, AND THE YIELD OF PROLINE FROM GLIADIN.

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TOWN [1928] outlined a method for the separation of the amino-acids by means of their Cu salts and for the isolation of proline in a state of purity: in the present paper modifications of the method are described and the yield of proline from gliadin as determined by isolation as the picrate is indicated.

Proline was first isolated by Fischer [1901] from the hydrolysis products of caseinogen by the ester distillation method. Later [1910] he improved the method of isolation, extracting the *dl*-proline with alcohol from the alkaline hydrolysate of gelatin and isolating it as the alcohol-insoluble Cu salt. This was the usual method of estimation of proline until Van Slyke suggested that it could be estimated by a determination of the non-amino-N in the alcohol-soluble portion of the amino-acids. This method gives results considerably higher than those of direct isolation; for example, in the case of gliadin, Osborne & Clapp [1906] obtained by isolation a proline content of 7.06 %, whereas later Osborne & Guest [1911] by Van Slyke's method found 13.2 %.

In the course of the present work six hydrolyses, each of 600 g. of protein, have been carried out. In the early hydrolyses, important findings were: (1) proline is difficult to extract with cold absolute alcohol, indeed the pure substance is only sparingly soluble; (2) proline is partly precipitated by phosphotungstic acid and this reagent therefore is not a specific precipitant for the bases [compare Thimann, 1930]; (3) proline can be effectively separated from other amino-acids by means of the solubility of its Cu salt in cold absolute methyl alcohol; (4) proline can be isolated in a pure state from this Cu salt, after decomposition, as the picrate.

A survey of the available literature indicates that the Cu salts of the amino-acids falls into three characteristic groups:

Group 1. Cu salts soluble in methyl alcohol and also in water: Proline, hydroxyproline, isoleucine, valine, the "hydroxyvaline" of Schryver and the peptide of phenylalanine and proline isolated from gliadin by Osborne.

Group 2. Cu salts insoluble in methyl alcohol but soluble in water: Glycine, glutamic acid, serine, alanine, arginine, histidine, lysine and also "hydroxy-glutamic acid" and the pyrrolidone compounds.

Group 3. Cu salts insoluble in water and in methyl alcohol: Leucine, phenylalanine, tyrosine, aspartic acid and cystine.

A fourth hydrolysis indicated that 98 % of the total N was accounted for in this gross separation of Cu salts. Only the methyl alcohol-soluble fraction of

the latter is considered in this paper. This fraction from the fourth hydrolysis yielded pure proline picrate by the earlier method [Town, 1928].

The methyl alcohol-soluble Cu salt fraction from the fifth hydrolysis (500 g. protein) was decomposed and the proline removed as its picrate (95 g.), the mother liquor being extracted with ether.

The *ether-insoluble fraction*, which still contained picric acid, was freed from the latter and reconverted into Cu salts which were extracted with methyl alcohol; the insoluble portion was rejected. The soluble portion was decomposed and the resulting alcoholic solution treated with alcoholic CdCl_2 to precipitate any residual proline. The filtrate, freed from Cd, was saturated with HCl gas and filtered from a small quantity (5 g.) of glutamic acid hydrochloride; removal of HCl left a syrup which contained no proline and from which nothing could be isolated.

The *ether-soluble fraction* was also converted into Cu salts and extracted with dry methyl alcohol. The alcoholic solution of the amino-acids recovered from the soluble Cu salts was precipitated with alcoholic CdCl_2 to remove traces of proline. The filtrate, after various unsuccessful attempts to precipitate it with nitro-compounds analogous to picric acid, was dried *in vacuo* and extracted with acetone in a Soxhlet apparatus. The large soluble portion contained diketopiperazines and was left for future examination. The acetone-insoluble residue gave Cu salts consisting of a very soluble minor fraction and a major fraction sparingly soluble in water; from the latter, after decomposition and fractional crystallization of the amino-acids from alcohol, there were obtained 1.43 g. leucine and 3.5 g. *isoleucine*. Confirmation of this isolation will be reported separately [compare Town, 1929].

The total yield of proline obtained in this hydrolysis was 37.7 g.—equivalent to 8.50 % of the dry protein—but it was obtained in three fractions as follows:

	g.
As proline picrate 	31.7
As CdCl_2 ppt. from ether-insoluble fraction ...	2.7
As CdCl_2 ppt. from ether-soluble fraction ...	3.3
	<hr/> 37.7

This yield of proline it is to be noted approximates more closely to that obtained by Osborne & Clapp [1906] by direct isolation than to the figure deduced by Osborne & Guest [1911] from determination of non-amino-N. This hydrolysis however suffered from two important defects. The first was that the separation of glutamic acid as water-soluble Cu salt was incomplete; although only 2.06 % of the glutamic acid came through in the wrong fraction, its presence rendered the isolation of the proline much more difficult. The second defect was that the proline was not completely precipitated as the picrate and also that the picrate has an appreciable solubility in ether; extraction of unprecipitated picrate with ether however removed also much troublesome diketopiperazine material.

The sixth hydrolysis, described in more detail below, was designed to overcome these defects. The dicarboxylates were removed from the H_2SO_4 -free hydrolysate as Ba salts insoluble in 75 % alcohol. The filtrate was concentrated at low temperature under reduced pressure to remove ammonia along with the alcohol, freed quantitatively from Ba and then converted into Cu salts. The methyl alcohol-soluble Cu salts were decomposed, the amino-acids taken up in 95 % alcohol and the proline precipitated with alcoholic CdCl_2 . Only the investigation of the CdCl_2 precipitate has been completed and is described here; the filtrate is being examined at present and the results will be communicated later.

The CdCl_2 precipitate was decomposed and the aqueous filtrate treated with picric acid, yielding 156 g. proline picrate. The mother-liquor from this yielded ether-insoluble and ether-soluble fractions of which the latter contained, besides picric acid, appreciable quantities of diketopiperazines which are being examined; the ether-insoluble fraction, containing 4.85 g. N, was subjected to Vickery's procedure for the removal of the basic amino-acids which preliminary tests had indicated to be present, after which there was finally obtained a further crop of 7.97 g. pure proline picrate.

The total yield of proline obtained in this hydrolysis is 55.0 g., obtained as follows:

	Wt. of picrate g.	Wt. of proline g.
1st crop	156.0	52.0
Final crops	8.0	2.7
	0.8	0.3
	<u>164.8</u>	<u>55.0</u>

This corresponds to 10.34 % of the dry protein, or expressed as proline N/total N to a yield of 7.32 % of the total N.

It seems important to the author that all results should be expressed in the latter manner, as otherwise unduly favourable figures are obtained for the yields of the protein hydrolysis products. The result obtained above is believed to represent with some accuracy the yield of proline from gliadin. It may be objected that the other fractions of the Cu salts or the CdCl_2 filtrate from the methyl alcohol-soluble Cu salt fraction still contain proline. These fractions however have now been examined in sufficient detail to prove the absence of proline; the results of this examination will be reported later. It will be noted that the yield of proline obtained above is higher than that previously obtained by direct isolation, but definitely lower than the figure obtained by merely determining the alcohol-soluble non-amino-N; this is due to the presence of peptides and diketopiperazines some of which are very resistant to hydrolysis. An important feature of this separation is that 94.6 % of the proline was removed in the first crops of picrate; the examination of the residue although occupying several months gave but little to increase the yield; moreover, the picrate obtained was pure and scarcely needed the one recrystallization which it was given.

EXPERIMENTAL.

600 g. gliadin containing 10.22 % moisture, 1.08 % of ether-soluble material and 15.27 % N were boiled under reflux for 36 hours with 30 % H_2SO_4 (510 ml. conc. acid to 2500 ml. water). The total N in the hydrolysed protein was 91.6 g. The hydrolysed solution was diluted with 5100 ml. water and the humin filtered off (8.6 g.; N, 0.3 g.). The filtrate was now treated with hot aqueous baryta until just acid to Congo red, the BaSO_4 being filtered and washed twice with boiling water (wt. of 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 bulky precipitate of barium dicarboxylates allowed to settle overnight before filtration. The examination of the dicarboxylates has been completed and will be described later.

The filtrate and washings from the dicarboxylates were freed from alcohol and ammonia by concentration under reduced pressure (bath at 50°). Removal of ammonia at this stage greatly facilitates the later separation of the Cu salts.

The concentrated solution (4600 ml.) was freed from Ba (wt. of BaSO_4 , 372 g.; N, 0.56 g.), further concentrated to 4 l. (N, 41.4 g.) and then evaporated on the water-bath with 200 g. of pure $\text{Cu}(\text{OH})_2$ until a thick viscous mass was obtained which was granulated with acetone in the manner previously described [Town, 1928]. After complete removal of acetone *in vacuo* over H_2SO_4 , the dry salts (565 g.) were extracted six times by shaking with 1.5 l. dry methyl alcohol for 2 hours. The residue was then similarly extracted four times with water. By the above fractionation the amino-acids have been divided up as follows:

	Total N in fraction (after removing metals) g.	% of total N
Dicarboxylates	24.61	26.9
NH_3 distilled from filtrate of dicarboxylates	24.10	26.3
Methyl alcohol-soluble Cu salts	24.82	27.1
Water-soluble Cu salts	8.60	9.4
Water-insoluble Cu salts	6.77	7.4
	88.90	97.1

It will be seen that no serious losses occur in this fractionation, for 97.1 % of the total N has been accounted for, and the remainder is mainly adsorbed on the BaSO_4 precipitate.

The methyl alcohol-soluble Cu salts (with which this paper is concerned) were dissolved in water and freed from Cu and the solution of the amino-acids was evaporated to small bulk (N, 24.82 g.). 4 l. 95 % alcohol were now added and 6 g.

of tyrosine which separated were filtered off. The solution was now treated with saturated 95 % alcoholic CdCl_2 to complete precipitation. Both precipitate and filtrate were dissolved in water and freed from Cd and Cl. The whole process is summarized diagrammatically above (p. 1840).

Examination of the CdCl_2 precipitate.

The aqueous solution (1 l.) of the fraction after removal of the Cd and Cl contained 13.62 g. N of which 26.5 % was amino-N. The solution was treated at boiling point with picric acid (163 g.) equivalent to the non-amino-N present; on cooling the solution set to a paste of crystals of proline picrate; these were collected and recrystallized from hot water. Weight of 1st crop, 138 g.; m.p. 153°. (Picric acid as nitron picrate 66.7 %; theory 66.6 %.)

The filtrate on concentrating to 500 ml. and cooling in the refrigerator yielded a 2nd crop which after recrystallizing amounted to 7.5 g.; m.p. 153°. (Picric acid 66.5 %.) On concentrating the mother-liquor to 200 ml. a 3rd crop was obtained; wt. after washing with ether and recrystallizing 9.0 g.; m.p. 153°. (Picric acid 66.7 %.) Further concentration to 100 ml. gave another 1.5 g. proline picrate the total weight so far obtained thus being 156 g., corresponding to 52.2 g. proline or 6.34 g. N. This is a yield of 9.8 % of the protein or 6.92 % of the total N. The solubility of proline picrate was determined at this stage; 50 ml. of a saturated solution at 26° on evaporation to dryness gave 1.096 g. proline picrate, corresponding to a solubility of 2.2 % at 26°.

The mother-liquor from the picrate was diluted with an equal volume of water and extracted with ether; the aqueous solution was then strongly acidified with H_2SO_4 , the precipitated picric acid filtered off (20 g.), and residual picric acid removed by extraction with ether. The final aqueous solution contained 4.85 g. N.

All the ether extracts were mixed, freed from ether, decomposed with an excess of H_2SO_4 , picric acid (22 g.) filtered off and the N determined; found 1.67 g. The N of the CdCl_2 precipitation thus accounted for is:

	g. N		g.
As proline *	6.34	Original N content	13.62
In mother-liquor from proline	4.85		
In ether extracts	1.67		
Loss	0.76		
	<u>13.62</u>		

The ether-extracted material on freeing from H_2SO_4 and evaporation *in vacuo* solidified; it was very soluble in water and gave a slight flocculent precipitate with phosphotungstic acid. Amino-N was 43.6 % of total N and the fraction contained appreciable quantities of diketopiperazines. It has not yet been systematically examined.

Examination of mother-liquor from proline picrate. This fraction (4.85 g. N) was evaporated to a syrup which was insoluble in acetone and alcohol but soluble in 60 % alcohol. Amino-N was 55 % of total N and was not increased on prolonged hydrolysis with conc. HCl, thus indicating the absence of peptides. The fraction gives no precipitate with alcoholic CdCl_2 , a small precipitate with mercuric acetate in 50 % alcohol and a heavy precipitate with phosphotungstic acid. It contains no ammonia or tyrosine and gives an amorphous Cu salt soluble in methyl alcohol. When treated according to Vickery & Leavenworth [1928] it yielded 0.68 g. histidine diflavinate, 6.0 g. arginine flavinate equivalent to 2.14 g. arginine and 0.71 g. lysine picrate. The recovery of these bases

was accompanied by appreciable losses of N, the nature of which could not be determined.

The residue not precipitated by phosphotungstic acid in the above separation, after freeing from precipitants, contained 2.76 g. N of which 61 % was amino-N. On evaporation it yielded a syrup insoluble in alcohol but soluble in 60 % alcohol; no crystalline picrate could be obtained from it. The solution in 60 % alcohol on standing in the ice-chest deposited 3.20 g. of well crystallized material containing 13.23 % N and identified as serine.

The filtrate from this on evaporating at room temperature deposited 2.66 g. of a material crystallizing in rosettes of needles containing 12.0 % N; M.P. 237°. This material is not valine, because although apparently homogeneous it yields no phenylisocyanate or 3:5-dinitrobenzoyl derivative. It is being further investigated.

After these substances had separated there was left a solution containing 1.82 g. N of which 50 % was amino-N. After preliminary experiments it was treated with 15 % mercuric acetate in 50 % methyl alcohol; the precipitate after decomposition gave a solution (0.19 g. N) from which after evaporation and treatment with alcohol there was obtained 0.54 g. more of serine.

The filtrate from this precipitation on diluting with twice its volume of 95 % alcohol deposited a further copious precipitate of mercury salt which was collected and decomposed. The solution (0.63 g. N; 68 % amino-N) was evaporated to a syrup from which, by long keeping with methyl alcohol, 0.69 g. of substance crystallizing in tetrahedra was obtained (N, 14.95 %; α -naphthylisocyanate M.P. 184°). An accident caused the loss of the rest of this fraction.

The residue, not precipitated by mercuric acetate, after freeing from reagent contained 0.92 g. N (36% amino-N) but gave only a syrup on evaporation. Picric acid equivalent to the non-amino-N was added and the mixture was left in the ice-chest; 7.2 g. proline picrate were obtained having m.p. 149°, raised to 153° after one recrystallization (picric acid 66.5%). The solution on repeated concentration gave further crops which were fractionally crystallized yielding 0.77 g. proline picrate and 2.33 g. sodium picrate which probably originated from the baryta used in the precipitation of the dicarboxylates.

The mother-liquor from these picrates, freed from picric acid, still contained 0.51 g. N. It was precipitated with methyl alcoholic mercuric acetate and from the non-precipitated portion 0.8 g. proline picrate, m.p. 149°, was isolated. No other identifiable component could be obtained. The results of the above series of operations are indicated in the above scheme (p. 1842).

DISCUSSION.

A balance sheet of the distribution of the N in the CdCl₂ precipitate of the methyl alcohol-soluble Cu salts appended below indicates clearly the substances which have been isolated as well as the losses of N which have been incurred.

N distribution in the CdCl ₂ precipitate.		Original N content
	N (g.)	
1st crops of proline picrate	6.34 g.	
Final crops of proline picrate	0.36 g.	6.70
Diketopiperazine material		1.67
Histidine		0.04
Arginine		0.69
Lysine		0.05
Losses in pptn. of bases		1.25
Serine		0.50
Substance with 12.0% N		0.32
Substance with 14.95% N		0.10
Loss due to accident		0.50
In final syrup		0.44
		<hr/> 12.26
Unaccounted for; i.e. lost on ppt. etc.		1.36
		<hr/> 13.62

Although the losses are high, it must be remembered that this examination was carried out under no standard plan, the method of attack having to be determined at each stage; it has been of an exploratory nature (the results have not been corrected for the material used in the N determinations). Also the examination of the proline fraction is well known to be difficult. The investigation shows that 49.2% of the N of the fraction is proline-N. ●

According to the literature the methyl alcohol-soluble Cu salts should be those of proline, hydroxyproline, isoleucine, valine and the peptide phenyl-alanylproline. Of these isoleucine and valine are not precipitated by alcoholic CdCl₂ and should be in the CdCl₂ filtrate. The investigation of this fraction has proceeded far enough to say that these amino-acids are definitely present and the details will be reported later.

The amino-acids serine, arginine, histidine and lysine have come through from the water-soluble Cu salt fraction to the extent of 13.7, 2.0, 8.0 and 29.2% respectively on the basis of the figures of Osborne *et al.* [1915] for the percentages of these amino-acids present in gliadin: thus the leakages into the wrong fractions are quite small, being serious only in the cases of arginine and serine. These

amino-acids also are not the main hydrolytic products of the protein. The investigation of the water-soluble and the water-insoluble fractions has proceeded far enough to indicate that they contain substantially the amino-acids to be anticipated. It therefore appears that the separation of amino-acids by means of their Cu salts may be of some permanent value and perhaps contribute to the elucidation of that fraction of the total N of proteins which is as yet unaccounted for, and which unfortunately is still a considerable proportion of the whole.

SUMMARY.

An investigation is being carried out to determine whether the differences in the solubilities of the Cu salts of amino-acids can be applied to effect their separation.

In the gross separation of the Cu salts into three fractions 97 % of the N can be accounted for.

The examination of the CdCl_2 precipitate obtained from the methyl alcohol-soluble Cu salts is described in detail.

The yield of proline from gliadin has been determined by direct isolation as the picrate, this amino-acid being the chief component of the CdCl_2 precipitate.

The yield obtained of 10.34% is believed to represent with some accuracy the proline content of gliadin. The figure is considerably higher than that previously obtained by isolation, but much lower than values obtained by determination of the non-amino-N. This is due to the presence of diketopiperazines, which have been isolated but not yet identified.

Other amino-acids, notably arginine and serine, which should belong to the water-soluble Cu salt fraction, find their way into the methyl alcohol-soluble Cu salt fraction to a small extent.

The examination of the CdCl_2 filtrate from the methyl alcohol-soluble Cu salt fraction is being continued and will be reported later.

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