XLIII. THE AMINO-ACIDS OF COCKSFOOT PROTEIN WITH SPECIAL REFERENCE TO THE DICARBOXYLIC ACIDS AND PROLINE.

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IN continuation of the research dealing with the amino-acid composition of cocksfoot protein, of which the basic amino-acids have already been determined [Miller, 1935], an account is now given of the isolation of proline by the method of Kapfhammer and Eck [1927], combined with that of Spörer and Kapfhammer [1930], and of the isolation of aspartic and glutamic acids by two slightly different methods both based on the procedures of Foreman [1914], Dakin [1918; 1920] and Jones and his co-workers (*e.g.* Jones and Moeller [1928]). No direct evidence for the presence of either hydroxyproline or hydroxyglutamic acid was obtained. During the course of the work the monoaminomonocarboxylic acids have been obtained in crystalline form by the usual butyl alcohol method of Dakin; their mean N content has been determined but the separation of the individual amino-acids is as yet incomplete.

It has already been suggested and partly confirmed that the low N content of the grass proteins prepared in this laboratory is due to the presence of N-free impurity [Miller and Chibnall, 1932; Chibnall *et al.*, 1933; Miller, 1935]. The analysis of cocksfoot protein is now sufficiently far advanced to permit calculation of the amount of this adulterant, and in a discussion at the end of this paper it has been deduced that the grass proteins, if they could be prepared free from non-nitrogenous impurities, would contain about 16 % N. This authenticates the assumption made by agricultural workers that the protein content of forage crops can be calculated from the "true protein" N by multiplying by the factor 6.25 (*i.e.* 100/16).

EXPERIMENTAL.

The dicarboxylic acids.

Exp. 1. 50 g. of a cocksfoot protein (AG), having a dry weight of 49 g. and N 13.2 % of the dry weight, were hydrolysed with 270 ml. of 20 % (1 : 1) HCl, by heating on a steam-bath for 1-2 hours and then boiling under reflux for 36 hours in an oil-bath. 5.34 g. of "acid humin", containing 3.80 % N, were separated. The clarified solution contained 6.16 g. N, making a total, with the humin-N, of 6.36 g. The latter figure will be referred to as "protein-N", on which all yields, in terms of N, will be calculated. The solution was concentrated to about 300 ml. (this and all subsequent distillations were carried out *in vacuo* below 50°) and treated with solid barium hydroxide until alkaline to litmus. The "alkaline humin" was filtered off, washed with hot water and dried at 105°. This weighed 5.10 g., and contained 2.23 % N. The total humin (acid + alkaline) weighed 10.44 g. and contained only 0.345 g. N; it represents therefore the major part of the non-protein impurity present in the protein preparation and by

difference the protein itself must have weighed 38.56 g. and have contained between 6.015 and 6.36 g. N, *i.e.* between 15.6 and 16.4 %. This point is developed in the discussion.

The amino-acid filtrate was concentrated to about 400 ml. and a Foreman precipitation carried out, but using barium hydroxide in place of calcium hydroxide since the barium ion was easier to remove. In this and all subsequent Foreman precipitations the procedure was as follows, with the exception of the volume of alcohol added and of certain other variations which will be stated in each case. The solution was saturated with barium hydroxide at room temperature by adding the solid material with shaking till a little remained undissolved and was then poured with stirring into 93 % alcohol (in this case, 5 vols.) and the mixture left at room temperature for 2 days. The insoluble barium salts were filtered off, washed once with alcohol and suspended in water, and the barium was removed exactly with H_2SO_4 . In this instance 2 l. of water were used in an attempt to dissolve the barium salts completely, but a small amount of an insoluble substance, containing 0.037 g. N, still remained. Later experience suggested that considerably less water would have effected the same result. The filtered solution, containing 1.63 g. N, was treated with 2.5 l. of alcohol and left at 0° overnight. The barium salts were decomposed as before to give a solution (solution A) containing 0.705 g. N. The alcoholic filtrate from this operation was evaporated at 40° to dryness to remove alcohol. The residue was practically all dissolved in 400 ml. of water, the solution treated with 500 ml. of alcohol, and left in the ice-chest overnight. The precipitated barium salts were decomposed as before; the resulting solution (solution B) contained 0.181 g. N; of which 87.2 % was amino-N. (Solutions A and B were worked up for glutamic acid as described later.)

This last alcoholic filtrate, after removal of solid material (1.9 g. probably largely barium carbonate with some tyrosine) which had been deposited while the solution had been standing, was concentrated at 40° to remove alcohol, and barium removed with H_2SO_4 . The solution contained 0.672 g. N (96 % amino-N), and 6.19 g. solids. It was concentrated to 100 ml. and a Foreman precipitation carried out, using $1\frac{1}{2}$ vols. of alcohol and leaving at 0° overnight. The barium precipitate was worked up in the usual way; the resulting solution (solution C) contained 0.092 g. N (all amino-N). The alcoholic filtrate was treated as before and another Foreman precipitate taken out from 50 ml. of solution, using an equal volume of alcohol; the solution (solution D) derived from the barium salts contained 0.0511 g. N.

Glutamic acid. Solutions A and B were combined, freed exactly from $\text{SO}_4^{=}$, and concentrated at 40° to 80 ml. After saturation at 0° with dry HCl, the solution was left in a glass-stoppered bottle for a week at 0°. The mass of crystals was collected on a sintered glass Gooch crucible, washed with a little cold concentrated HCl and dried in a vacuum desiccator over NaOH sticks: yield 1.13 g. The filtrate was concentrated to 25 ml., resaturated with HCl and a second crop (1.92 g.) obtained. The mother-liquor was further concentrated to 15 ml., resaturated as before and a third crop (2.73 g.) procured. At this stage solutions C and D were added to the mother-liquor, leading to a fourth crop (0.51 g.). The total yield of glutamic acid hydrochloride, allowing for aliquots withdrawn for analyses, was 6.73 g., or 5.39 g. of the free amino-acid. This represents a yield of 13.1 % of the original weight of protein (recalculated on a basis of 16 % N); or, in terms of N, 8.03 % of the protein-N. The recrystallised product contained 7.68 % N; calculated for glutamic acid hydrochloride, 7.61 %. The ash was negligible.

The isolation of aspartic acid in this experiment will not be described in detail since the yield was low, probably owing to certain unnecessary steps in the procedure, the acid being precipitated successively as its barium salt (Foreman precipitation), as its lead salt [Dakin, 1918] and finally isolated as the copper salt.

Exp. 2. 100 g. of cocksfoot protein (AB), having a dry weight of 94.4 g. and N 13.7% of the dry weight, were hydrolysed as in the previous experiment. 11.24 g. of humin, containing 3.93% N, were obtained. The solution contained 12.26 g. N, or with the humin-N the total would be 12.70 g. ("protein-N"). The solution was freed from Cl^- with silver sulphate, and the excess Ag^+ removed by means of H_2S . The excess of the latter was removed by concentration; the solution then contained 11.91 g. N, a loss of 0.35 g. N. H_2SO_4 was quantitatively removed with barium hydroxide solution, resulting in a solution reacting just acid to litmus. The barium sulphate was centrifuged off and digested three times successively with water on a boiling water-bath. The solution and washings were concentrated at 45° to about 700 ml. (if the volume was reduced lower than this a solid crystalline mass resulted) and left overnight. The cream coloured solid was filtered off, washed and dried at 105° . It weighed 3.25 g., contained 7.39% N, and apparently consisted chiefly of tyrosine and leucine. In Table I, given later, this product is referred to as monoamino-acid mixture A.

The filtrate, containing 11.40 g. N, was concentrated to 350 ml., divided equally into four 500 ml. centrifuge bottles stoppered with rubber bungs and shaken for 10 min. in a shaking-machine with 1200 ml. of *n*-butyl alcohol. The liquids were then centrifuged and the clear upper layer carefully syphoned off and concentrated at 40° under reduced pressure. The aqueous phase remaining was further extracted with butyl alcohol as before. After this second extraction the aqueous solution was made neutral to litmus with barium hydroxide solution. (This was an improper step as the glutamic acid hydrochloride subsequently isolated contained barium chloride.) The solution was then concentrated to about 300 ml. (75 ml. in each bottle) and given 8 further extractions, using 5 vols. of butyl alcohol. The aqueous phase, now containing 6.87 g. N (54.1% of the protein-N), was concentrated to 200 ml. (50 ml. in each bottle) and further extracted 5 times with 8 vols. of butyl alcohol.

The butyl alcohol extracts were concentrated to about 500 ml. and the cream coloured amino-acids filtered off and washed with butyl alcohol followed by acetone. They weighed 38.0 g. and contained 11.9% N. This material is referred to in Table I as monoamino-acid mixture B. The butyl alcohol filtrate contained 0.525 g. N consisting largely of proline-N.

Glutamic acid. The aqueous phase, containing 5.91 g. N. (46.5% of the protein-N), was concentrated to about 130 ml., saturated with dry HCl at 0° and glutamic acid hydrochloride isolated in the usual way: 10.2 g. (in 3 crops) were obtained. The mother-liquor was repeatedly evaporated to a syrup from water to remove free HCl, taken up in water and made slightly alkaline to litmus with solid barium hydroxide. The small amount of humin was removed by filtration, the filtrate concentrated to 100 ml. and a Foreman precipitation carried out using 10 vols. of alcohol and leaving overnight. The alcohol filtrate (1), containing 2.09 g. N, was treated later. The barium salts were worked up as usual; the resulting solution, containing 2.605 g. N (equivalent to 20.5% of the protein-N), was concentrated to a thin syrup and saturated with HCl at -10° : 2.74 g. of glutamic acid hydrochloride were obtained. In this experiment then a total of 12.94 g. had been collected. It contained 13.5% of ash (for the reason stated previously) and 3.5% of ammonia-N; the N-content of the ash- and ammonia-

Table I.

	Yield		Actual wt. isolated g.	N in amino- acid %	*
	Expressed as N in % of protein-N	As % of total wt. of protein (N, 16.0%)			
Arginine	13.6	6.11	5.62	32.2	2.520
Histidine	1.66	0.89	0.82	27.1	0.309
Lysine	5.27	3.98	3.67	19.7	1.007
Proline	2.0	2.52	2.21	12.17	0.374
Glutamic acid	8.03	13.10	10.12	9.5	1.340
Aspartic acid	3.25	5.32	4.30	10.5	0.629
Monoamino-acid mixture A	1.9	4.04	3.26	7.42	0.337
Monoamino-acid mixture B	35.6	47.0	38.0	11.9	6.290
Monoamino-acid mixtures C and D	3.29	3.64	2.94	14.3	0.585
Ammonia	6.0	1.15	0.93	82.3	1.069
(Humin)	5.4	—	—	—	—
Total	86.00	87.75	71.87	—	14.460

* % of N contributed by each amino-acid (or mixture): e.g. for arginine, $\frac{5.62 \times 32.2}{71.87} = 2.520$.

free hydrochloride was 7.85%. Calculated from this N content the yield of free glutamic acid was 11.5% of the original weight of protein (N, 16.0%), or in terms of N, 7.05% of the protein-N.

Aspartic acid. The mother-liquor from the above hydrochloride, after removal of free HCl by concentration, contained 2.35 g. N (87.2% amino-N). It was concentrated at 40° to about 100 ml. and another Foreman precipitation carried out, using 1½ vols. of alcohol and leaving 1 hour at 0°. The alcoholic filtrate (2), containing 1.175 g. N, was treated later. The barium salts gave a solution containing 1.055 g. N. The solution (volume 250 ml.) was heated to boiling and about 10 g. of Kahlbaum's cupric hydroxide added; the excess was filtered off, washed with hot water, and the filtrate and washings were left overnight at 2°. The copper aspartate was filtered, washed with a little cold water and dried at 105° overnight: weight, 6.28 g. This is a yield of free aspartic acid of 5.32% of the original weight of protein (N, 16.0%); or expressed as aspartic acid-N, 3.25% of the protein-N. Analyses were done on the air-dry copper aspartate (containing 4½H₂O), since it was found difficult to render the salt anhydrous. After one recrystallisation the following values were obtained: N, 5.13; Cu, 22.4%. C₄H₅O₄NCu, 4½H₂O requires N, 5.23; Cu, 23.0%.

The filtrate on concentration yielded no further crop of copper aspartate; it contained 0.547 g. N, of which 85.2% was amino-N. Since the N in the solution was not all in the amino-form, it was thought that basic amino-acids might be present. The solution (250 ml.) was therefore treated with 18 ml. of concentrated HCl followed by 20% phosphotungstic acid in 3% HCl solution until precipitation was complete. The precipitate was filtered off, washed with the usual reagent, and the filtrate and washings were freed from phosphotungstic acid with the usual 1:1 amyl alcohol: ether mixture. The aqueous solution was evaporated to dryness under reduced pressure and the residual syrup dissolved in water. The solution contained 0.446 g. N (all amino-N). No further amounts of glutamic and aspartic acids could be separated by the usual methods and an attempt to isolate hydroxyglutamic acid by Dakin's procedure [1918] was unsuccessful.

Monoamino-acid mixtures. The alcoholic filtrates (1) and (2) mentioned above were combined, concentrated at 40° to remove ammonia and alcohol, taken up

in water, and freed from barium with a slight excess of H_2SO_4 . The resulting solution, containing 3.27 g. N, was treated, in a volume of 1.5 l., with 20 % phosphotungstic acid in 5 % H_2SO_4 to complete precipitation, in order to remove bases. The mixture was left overnight, centrifuged and the precipitate washed with 3 % phosphotungstic acid (in 5 % H_2SO_4). The supernatant solution and washings, containing 1.073 g. N, were freed from phosphotungstic acid by making alkaline with hot barium hydroxide solution, filtering off the precipitate and washing with alkaline water. Barium was removed from the filtrate, which then contained 0.880 g. N (86.5 % amino-N). All Cl^- was next removed with silver oxide and the excess Ag^+ with H_2S . After concentration the solution then contained 0.841 g. N. It was concentrated to 50 ml. and extracted 10 times with 4 vols. of butyl alcohol. The solid monoamino-acids obtained from the extract weighed 1.94 g. and contained 14.3 % N. In Table I, this material is referred to as monoamino-acid mixture C.

The aqueous phase, containing 0.378 g. N, gave no Foreman precipitate on a small sample; it was therefore further treated with 20 % phosphotungstic acid in 5 % H_2SO_4 solution and the copious precipitate removed on the centrifuge and washed twice with the usual reagent. The clear solution and washings, containing 0.298 g. N, were freed from phosphotungstic acid with barium hydroxide solution and then exactly from barium with H_2SO_4 . The solution (0.253 g. N) was then concentrated to 8 ml., 3 vols. of alcohol added and the turbid solution left for some days in a refrigerator. The white solid was filtered off and dried in a vacuum desiccator over phosphorus pentoxide: yield 0.996 g., containing 14.22 % N, and therefore probably representing approximately the same mixture of amino-acids (including glycine, alanine and possibly serine) as mixture C. This is referred to in Table I as monoamino-acid mixture D.

Proline.

To obtain some idea of the maximum quantity of proline present in cocksfoot protein the following experiment was performed.

Exp. 3. The same protein (AB) was used as in the previous experiment. 2.078 g. were hydrolysed by boiling with 35 ml. of 20 % HCl for 22 hours. The humin contained 0.010 g. N and the filtrate 0.255 g., so that the protein-N was 0.265 g. The filtrate was made alkaline with a slight excess of an aqueous suspension of magnesium oxide and ammonia and humin removed in the usual way. The solution then contained 0.239 g. N, of which 0.201 g. was amino-N (including the $\epsilon\text{-NH}_2$ group of lysine). Thus the total non-amino-N was 0.038 g., or 14.34 % of the protein-N; this will include the non-amino-N of proline and hydroxyproline, arginine, histidine and possibly some diketopiperazine. Now this particular cocksfoot protein had not been analysed for the basic amino-acids, but assuming it to contain the same percentage as the cocksfoot protein used in the large scale analysis described previously [Miller, 1935], the non-amino-N due to arginine and histidine would be 11.31 % of the protein-N. Thus the maximum amount of proline-N and hydroxyproline-N (including any diketopiperazine-N) would be 3.03 % of the protein-N.

For the quantitative isolation of proline the following method [Kapfhammer and Eck, 1927; Spörer and Kapfhammer, 1930] was adopted.

Exp. 4. For convenience the actual solution from which the basic amino-acids had already been removed by the Vickery large scale analysis [Miller, 1935] was used, although it should be realised that the latter involved losses which might conceivably have included small amounts of proline and hydroxy-

proline. The filtrate from the lysine phosphotungstate was freed from phosphotungstic acid by means of barium hydroxide and then from excess of the latter by the addition of H_2SO_4 to give a reaction slightly acid to Congo paper. The final solution was concentrated at 45° to 150 ml., when solid began to separate. The mixture was cooled in ice to freeze out as much of the material as possible, and filtered. The dried precipitate weighed 8.42 g. On concentration of the filtrate 2.88 g. more were obtained, making 11.3 g. in all. The combined precipitates contained 10.0% N and probably therefore consisted largely of leucine (N, 10.69%). The filtrate was concentrated to 120 ml. and treated at 60° with an 8% solution, also at 60° , of 30 g. of ammonium reineckate (recrystallised from water at 60°). On cooling crystallisation began and was allowed to proceed to completion in the refrigerator overnight. The precipitate was filtered off, washed with a little ice-cold water and dried over phosphorus pentoxide *in vacuo*: yield 23.5 g. On concentration of the filtrate and addition of more ammonium reineckate solution 11.5 g. more were obtained, making a total of 35 g. The latter was suspended in 300 ml. of 50% methyl alcohol, warmed, and a hot solution of 60 g. of copper sulphate (A.R.) added with stirring. The brown precipitate was filtered off, washed, and the filtrate saturated with sulphur dioxide and left overnight. The precipitate was filtered off, washed, and the filtrate concentrated to small bulk. To this hot solution was added silver sulphate to remove the remainder of the CNS^- , and then after cooling, H_2S was passed to remove Cu and Ag. The resulting solution was concentrated to 300 ml., 50 g. of barium hydroxide added and all ammonia distilled off *in vacuo*. The precipitate was centrifuged off, washed and the liquid freed from barium with H_2SO_4 . The solution then contained 0.556 g. N, of which 0.232 g. was amino-N; *i.e.* 0.324 g. of non-amino-N, or 2.76% of the protein-N. The solution was evaporated to dryness at 45° , and the solid residue triturated with hot alcohol and left overnight. The precipitate was filtered off, washed with absolute alcohol and dried over phosphorus pentoxide in a vacuum: weight 1.243 g. It contained 10.0% of N, all of which was amino-N, and therefore did not contain any hydroxyproline, which would normally appear at this stage if present in appreciable amount in the original protein.

The filtrate was evaporated to a syrup which was dissolved in about 90 ml. of 50% alcohol and treated with 100 ml. of a 5% solution of cadmium chloride in alcohol. The solution was left in the ice-chest overnight; the crystals which had then separated were filtered off and the filtrate concentrated to 50 ml. More cadmium chloride solution (50 ml.) was added and the liquid left in the ice-chest over a week-end. A second crop was isolated giving a total of 5.06 g. (Found: N, 4.65. $\text{C}_5\text{H}_9\text{O}_2\text{N} \cdot \text{CdCl}_2$ requires N, 4.71%.) Expressed as weight of proline this represents a yield of 2.52% of the original weight of protein (N, 16.0%); or in terms of proline-N, 2.01% of the protein-N. An attempt was made to improve this yield by adopting Bergmann's method [1935], but pure proline rhodanilate could not be isolated, probably owing to the large amount of humin and relatively small proportion of proline present in the grass proteins as compared with gelatin.

DISCUSSION.

The results of the foregoing experiments in which the amino-acids (or mixtures of them) have been actually isolated, are given in Table I, along with the basic amino-acids [Miller, 1935] and ammonia. Reading from left to right, the first two columns of figures give the yields of the various amino-acids; the last three columns are entered in order to arrive at the total mean percentage of

N in the sum of all the isolated amino-acids (or mixtures), *viz.* 14.46 %. (Humin with its low N-content has been purposely excluded from this calculation because the N-free impurity present in the original protein is concentrated in this fraction.) Now when a protein is completely hydrolysed, a water molecule is added at every peptide (and amide) linkage forming an α -amino-group (and ammonia). Of the above 14.46 %, 11.99 % is α -amino-N (including ammonia-N); *i.e.* $\frac{18 \times 11.99}{14} = 15.4$ % of water molecules have been added on to the original weight of protein in the formation of the amino-acids. Thus when this water is allowed for, the N-content of the protein, if non-nitrogenous adulterants be excluded, would be 17.1 %. Of course this value is calculated on a total yield of amino-acids of 86.0 %. The remaining 14 % of amino-acids would almost certainly contain a lower percentage of N than this, but even assuming an average figure of 10 %, the calculated N content in the pure cocksfoot protein (and experience suggests the proteins of most other common grasses also) would still be 16.0 %. This value is in agreement with that calculated previously on the humin-free protein (AG). Accordingly for purposes of comparison with the N contents of other practically pure plant proteins such as edestin (18.6 %), zein (16.1 %), proteins from the leaves of spinach (16.25 %) and alfalfa (15.7 %), a general figure of 16 % N for pure grass proteins can be safely assumed and this value has been adopted throughout this paper in calculating the yields of amino-acids in percentages of the total weight of protein.

The total N recovered in the amino-acids (although in some cases as mixtures not yet separated) so far isolated from the grass proteins, *viz.* 86.0 %, compares favourably with the corresponding figures for edestin, 84.4 %; and caseinogen, 82.3 %.

SUMMARY.

Glutamic acid, aspartic acid and proline have been isolated from cocksfoot protein in the following respective yields: 13.10, 5.32 and 2.52 % of the protein; or in terms of N, 8.03, 3.25 and 2.0 % of the protein-N. The presence of hydroxyglutamic acid and hydroxyproline could not be demonstrated.

Evidence is adduced that the protein, if it could be prepared pure—as yet impracticable—would contain about 16 % N.

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