CCLXXVIII. THE BASIC AMINO-ACIDS OF TYPICAL FORAGE GRASS PROTEINS.

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(Received August 28th, 1935.)

THE need for accurate information as to the quantities of amino-acids yielded on acid hydrolysis by proteins derived from green forage crops requires no emphasis in view of their value to the animal as food. Particularly important are arginine, histidine and lysine, the last being one of the two indispensable amino-acids which the animal body apparently cannot synthesise. This is especially exemplified in milk production from cows, for, the milk proteins being relatively rich in lysine, the milk yields will be low if insufficient of this amino-acid be present in the pasturage [*cf.* Morris and Wright, 1933, 1, 2].

In the present research the proteins from a number of typical forage grasses (including red clover) have been analysed for the basic amino-acids by methods which involve their actual individual isolation and which can therefore be expected to give reliable results. Estimation by the Van Slyke procedure, which depends mainly on the amount of nitrogen precipitated by phosphotungstic acid under controlled conditions, has been explored and found to give values for these proteins which can only be regarded as extravagant.

EXPERIMENTAL.

The preparation of the proteins used in the present work has already been described [Miller and Chibnall, 1932; Chibnall *et al.*, 1933].

Large scale analysis of the basic amino-acids of cocksfoot protein.

The method adopted was that of Vickery and his co-workers [*cf.* Vickery and Shore, 1932].

88.7 g. of dry protein (N, 13.9 %, ash-free) prepared from samples of cocksfoot cut in the spring of 1932 were used. The general procedure has been given in the above-mentioned and preceding papers [especially Vickery and Leavenworth, 1928], but since it has been varied from time to time in certain details it may be stated here that the following reagents were used in the present work:

1. Hydrochloric acid in the initial hydrolysis.
2. Silver oxide throughout, both for the introduction of excess silver ion and for the removal of chlorides.
3. Hydrogen sulphide to remove silver.
4. Kahlbaum's cupric hydroxide to precipitate cystine (present only in small amount in the grass proteins).
5. Kahlbaum's phosphotungstic acid for the precipitation of lysine.

The results are shown in Table I.

As a check on the total bases estimated by this and the Block procedure detailed below, a determination of total basic N by the usual method of Hausmann was also made, using again Kahlbaum's phosphotungstic acid.

Table I. *Large scale analysis of cocksfoot protein.*

	Expressed as N in % protein-N	Expressed as % of protein (N = 16.0 %)	% of S in flavianates and explosion points of picrates	
			Found	Theory
Arginine	13.6*	6.40	6.50	6.56
Histidine	1.66	0.93	8.23	8.17
Lysine	5.27	4.11	1st crop 267° 2nd and 3rd crops 260°	266-267°
Total bases	20.53	11.44		
Total bases (Hausmann)	20.0			

* This figure includes a solubility correction for the silver salt of arginine; if this correction be excluded the figure would be 12.7%.

Small scale analysis of the basic amino-acids of various proteins.

The method was essentially the very convenient application of the above large scale analysis to small quantities of proteins, devised by Block [1934]. His procedure was adhered to, but in addition, from the present author's experience, the following comments are worth recording.

In the initial removal of sulphuric acid as barium sulphate it was considered safer, in view of the bulkiness of the precipitate, to give it more than one washing with boiling water, especially in the case of proteins such as those from grasses, which give rise on hydrolysis to a considerable amount of humin.

Histidine. In the precipitation of histidine as its silver salt, it is more accurate and takes little longer after experience has been gained to follow the large scale analysis in the adjustment of the reaction to p_H 7.2, viz. withdrawing 2 ml. samples from the supernatant liquid after the precipitate has settled, mixing with 2-3 drops of bromothymol blue and comparing with 2 ml. of a buffer solution of p_H 7.2 with the same amount of indicator.

Lysine. Block recommends that the strongly alkaline filtrate from the precipitated arginine silver should be made acid to p_H 1 to remove barium. The present author has sometimes found it necessary to make more acid than this to ensure the removal of all barium, owing to the presence of a considerable amount of nitric acid. As Block implies it is advisable to precipitate the silver as sulphide before removing the barium sulphate precipitate, as the combined precipitates can then be washed more easily and with less chance of formation of colloidal solutions; if these do form they can be cleared by the addition of a drop or two of 20% sulphuric acid. Two washings were given. The resulting filtrate and washings should on no account be reduced in volume below 5 ml. otherwise the concentration of the nitric acid will bring about decomposition of the lysine.

Kahlbaum's phosphotungstic acid was used to precipitate the lysine. The lysine phosphotungstate should not be exposed to a high temperature for longer than is necessary, owing to the possibility of decomposition. For a similar reason, the solution, after the addition of the 5 g. of barium hydroxide, should not be heated for more than a few minutes at 90°. If decomposition has taken place at this or any previous stage it will be shown by the development of a yellow colour in the solution, either at once or after the addition of barium carbonate and subsequent concentration of the solution. This will result in the separation of a brown oil on adding alcohol to the concentrated lysine carbonate solution. If for any of the above reasons the lysine estimation be vitiated, the experiment can be repeated on another sample of protein without the necessity of precipitating arginine and histidine separately; they can be removed together as their silver salts at p_H 14. Results of the analysis are given in Table II, and when comparing them with those given in Table I it is to be remembered that no correction for the solubility of arginine silver has been applied.

Of the two methods described above that due to Vickery takes the longest time but would appear to be the most reliable and accurate. From Table I it will be seen that the flavianates analyse for sulphur to within less than 1% of the

Table II. *Small scale analysis of various proteins.*

Protein	Season when prepared	% of N	Expressed as N in % of protein-N				Hausmann total base
			Arginine	Histidine	Lysine	Total base	
Cocksfoot	Spring	13.9	12.2	2.2	*	—	20.0
Perennial rye-grass	Autumn	12.8	12.6	2.7	5.3	20.6	22.5
Hard fescue	Autumn	15.0	12.1	2.3	5.3	19.7	22.8
Timothy	Autumn	13.8	12.2	2.4	4.5	19.1	23.5
Red clover	Autumn	12.8	12.4	2.3	4.4	19.1	22.8
Rough-stalked } meadow grass }	Autumn	13.4	12.7	2.0	4.5	19.2	20.5
	Spring	13.7	13.1	1.9	5.3	20.3	23.1

* Fraction lost.

theoretical. The one possible source of error is in the correction for the solubility of the silver salt of arginine. This is of considerable magnitude, amounting to more than 6.5 %, and obviously cannot be fixed with any accuracy since the solubility will vary with the nature and quantity of the other amino-acids present.

The Block application of the above method is much more rapid and can be carried out on much smaller amounts of protein, but the time saving is mainly effected by decreasing the number of silver precipitations, for instance, giving only one histidine precipitation at p_H 7.2 instead of the two which Vickery and Leavenworth [1928] hold to be necessary in order to avoid arginine contamination in the final histidine fraction. Moreover Block takes out the histidine silver precipitate from a solution containing all the other products of protein hydrolysis, instead of taking out an initial silver precipitate at p_H 13–14 containing both arginine and histidine and separating these subsequently. At any rate in the series of Block analyses given in Table II the present author has found that both the arginine and histidine flavianates show sulphur contents varying from 2.5 to 8 % higher than the theoretical. Otherwise the agreement between the one large scale analysis (Table I) and the corresponding small scale analysis (Table II) is fairly close, the latter being a little lower in arginine and a little higher in histidine. This seems to point to arginine contamination in the histidine fraction, but this may not necessarily be so since the sulphur content of the histidine diflavianate is high. Moreover none of the characteristic orange-coloured arginine flavianate could be detected in the yellow histidine diflavianates. It is possible then that the high sulphur percentage is accounted for by slight contamination with flavianic acid itself, presumably due to the very small volumes of solutions used.

It will also be apparent from Table II that the yields of total bases by isolation fall short of the Hausmann figures by some 6–18 %, but part of this difference is accounted for because in the Block (and also the Vickery) method the figures given for lysine (isolated as picrate) represent yields of 69–77 % of the nitrogen in the fraction precipitated by phosphotungstic acid. If this residual nitrogen, presumably mainly non-lysine-N, is taken into account, the difference between the Hausmann figures and those obtained by isolation is reduced to 1–8 %. Taken as a whole, the results given in Tables I and II compare favourably with those quoted by Osborne [1924] for a large number of seed proteins analysed by the Hausmann and the older Kossel methods respectively.

Van Slyke analysis of the basic amino-acids of cocksfoot protein.

As has already been mentioned the total basic N calculated from the yields of the isolated bases is very close to that obtained by simple precipitation with phosphotungstic acid according to the procedure of Hausmann, but is considerably lower than the value (27.9%) obtained by Miller and Chibnall [1932] from a different sample of cocksfoot protein analysed by the method of Van Slyke. Since a sample of the protein used in the two former determinations was not available another sample containing 13.7% N was analysed by the latter method, and the total basic N (28.8%, uncorrected for solubility of bases, as shown in Table III) was even higher than that found by Miller and Chibnall.

Table III. *Van Slyke analysis of a cocksfoot protein.*

Expressed in % of protein-N uncorrected for solubility of bases.

Amide-N	...	...	...	6.28
Humin-N	...	...	...	6.14
Arginine-N	...	...	...	13.6
Histidine-N	...	...	...	7.1
*Lysine-N	...	...	...	8.1
Monoamino-N	...	...	...	57.6
Non-amino-N	...	...	...	0.25
Total recovered	...	...	...	99.07
Total basic N	...	...	...	28.8

* The cystine-N of this protein is about 0.15%.

Reference to the results of analyses of numerous seed proteins by the Kossel and Hausmann methods quoted by Osborne [1924] and the corresponding data for Van Slyke analyses collected by Larmour [1928] shows that discrepancies of this order, viz. 6-8 units in the percentage of basic N, are fairly general between the direct and indirect methods of analysis. In all cases the difference is accounted for almost entirely by enhanced values for histidine-N and lysine-N. Attention has occasionally been drawn to this point by previous workers, but the only constructive criticism so far advanced has been that of Gortner and Sandstrom [1925] who point out the possible interference of proline in the Van Slyke analysis. One important difference however in the respective procedures of Hausmann, Kossel, Vickery and Block on the one hand and of Van Slyke on the other hand seems to have passed unnoticed, namely, that in the former the precipitation of the bases with phosphotungstic acid is made in the presence of sulphuric acid, whilst in the latter it is made in the presence of hydrochloric acid. To find out if the change of mineral acid was indeed the cause of the discrepancy, the following experiment was carried out with a sample of the cocksfoot protein used in the second Van Slyke analysis.

Determination of N precipitated by phosphotungstic acid under Hausmann and under Van Slyke conditions.

4.09 g. of protein, containing 0.557 g. of N, were hydrolysed by boiling under reflux with 120 ml. of 20% hydrochloric acid for 16 hours. The solution was evaporated to dryness *in vacuo* at 45° to remove free hydrochloric acid, the residual syrup taken up in 300 ml. of water, and 45 ml. of a 17% aqueous suspension of magnesium oxide were added. Ammonia and humin were removed in the usual way and the amino-acid filtrate was reduced in volume to 100 ml. to provide four equal portions for the following experiments.

1. Two portions were treated according to Van Slyke's procedure as follows. Each portion was diluted to about 40 ml. and treated with 6 ml. of concentrated HCl and 5 g. of Kahlbaum's phosphotungstic acid dissolved in a little water. The mixture was diluted to about 67 ml., heated on a boiling water-bath until the precipitate had practically all dissolved and kept for 46 hours at room temperature. The recrystallised phosphotungstates were filtered off on a 5 cm. Büchner funnel through a hardened filter-paper and washed, without interrupting suction, with 17 ml. of the usual wash-liquid (2.5 % phosphotungstic acid in 3.5 % HCl) in portions of 4–5 ml. at a time. The whole of the precipitate was dissolved in *N* NaOH and submitted to Kjeldahl analysis.

2. The remaining two portions were each diluted to 100 ml. and treated with 5 g. of concentrated H_2SO_4 and 30 ml. of 20 % phosphotungstic acid in 5 % H_2SO_4 . The mixture was left for 22 hours at room temperature, centrifuged and the precipitate washed with 200 ml. of 2.5 % phosphotungstic acid in 5 % H_2SO_4 in three successive portions of 60–70 ml. each. The final centrifuged precipitate was analysed for total N as before.

In 1 (Van Slyke conditions) the duplicate results for total basic N were 29.4 and 29.8 % of the protein-N, whilst in 2 (Hausmann conditions) the corresponding figures were 22.4 and 22.4 % respectively. The experiment demonstrates quite clearly that the greatly enhanced values for total basic N obtained by the Van Slyke method are due to the employment of hydrochloric acid at this stage. The nature of this excess basic N is already being investigated in this laboratory, for clearly the present findings cast doubt on the validity of the whole Van Slyke procedure and of the numerous analyses of proteins and foodstuffs which have been made by means of it during the past 20 years.

The basic amino-acids of pasture proteins.

From Tables I and II it will be apparent that the proteins from the various forage grasses, including red clover, differ very little in their contents of basic amino-acids in spite of their variations in total N (12.8–15.0 %), confirming the view already expressed [Miller and Chibnall, 1932; Chibnall *et al.*, 1933] that the N-free material always present to some extent in these preparations is merely an adulterant, and that the leaf protein as a whole, though differing somewhat in each individual case, must be, if not a chemical entity, at least a mixture of proteins of very similar constitution.

In the two papers referred to immediately above evidence was given that the samples of protein, obtained in yields of from 10 to 30 %, could be taken as representative of the whole leaf protein. Since the basic amino-acids probably undergo little, if any, decomposition during the hydrolysis of a protein in the presence of much carbohydrate material, opportunity has been given to test this supposition in the following way.

A supply of freshly cut spring pasturage of good agricultural quality, consisting chiefly of perennial rye grass, was obtained from Jealotts Hill on the 22nd of May, 1935. The dry weight was 22 % and the total nitrogen 2.63 % of the dry weight. 8 kg. of the fresh grass were treated by the method of Chibnall *et al.* [1933] and 52.7 g. of protein (N, 12.1 %, ash-free) were obtained, representing a yield of 17.4 % of the total leaf protein.

The remainder of the fresh grass was dried rapidly (1–2 hours) in a steam-oven through which a slow current of air was drawn and then ground to a fairly fine powder in a small mincing machine. 29.2 g. of this material (dry weight, 26.9 g.) were exhaustively extracted with ether in a Soxhlet apparatus to remove fatty substances, chlorophyll *etc.* and the residue was treated twice successively

with 300 ml. of the following solvents at the boiling-points (to remove all non-protein nitrogenous substances), *viz.* 95 % alcohol, 50 % alcohol and water; 20 % of the total leaf-nitrogen was removed in this way. The final protein-rich residue was only faintly straw-coloured. Previous experience had shown that the protein was best extracted from this residue by the use of 4 % HCl; accordingly 13.5 g. of the dry residue, containing 0.45 g. N, were extracted with 450 ml. of 4 % HCl by boiling under reflux for 48 hours. After cooling and filtering, the dried residue weighed 5.34 g. and contained 0.053 g. N, whilst the filtrate and washings contained 0.373 g. N; 88 % of the total protein-N had therefore been extracted. This yield compares very favourably with the previously mentioned 17.4 % for the isolated protein. On the other hand the latter contained 12.1 % of N, whereas the protein extract contained solid material amounting by difference (13.5-5.34) to 8.16 g. with a nitrogen content of 0.373 g. or only 4.7 %.

The 4 % HCl extract mentioned above was evaporated to dryness under reduced pressure at 45°, the residue dissolved in water and the solution again concentrated to remove most of the free HCl. The residual syrup was dissolved in 40 ml. of water, an equal volume of concentrated HCl was added, the whole boiled under reflux overnight (16 hours) and the solution freed from HCl by evaporation *in vacuo* as described above. The final syrup was dissolved in water, the humin filtered off, washed and submitted to Kjeldahl analysis. It contained 2.65 % of the nitrogen in the initial 4 % HCl solution. The filtrate and washings (volume approx. 200 ml.) were treated with 1 ml. of concentrated H₂SO₄ and then with a suspension of silver oxide until free from chlorine. The precipitate was centrifuged and washed twice with 200 ml. of boiling water containing 1 ml. of concentrated HCl and finally with boiling water alone. The combined washings were again freed from chloride with silver sulphate (as no silver oxide was available), the silver chloride filtered off and washed once. Both these precipitates of silver chloride will probably contain a small amount of histidine silver which cannot be removed very readily in such a small scale experiment; it is to be expected therefore that the ultimate value for histidine-N obtained later will be slightly low. The combined filtrates were concentrated to 50 ml. and warm barium hydroxide solution was added carefully to p_H not > 4. The precipitate of barium sulphate was centrifuged and washed and the filtrate and washings were concentrated to 25 ml. At this stage the Block procedure described above was applied.

The results of the analysis are given in Table IV, together with that of the isolated protein. It will be seen that in spite of the relative large difference

Table IV. *Comparison of the basic amino-acids of the isolated protein and of the protein extracted by 4 % hydrochloric acid from spring pasture.*

	Expressed as N in % of protein-N			
	Arginine	Histidine	Lysine	Total base
Isolated protein	11.0	2.22	5.05	18.27
Protein extracted by 4 % hydrochloric acid	10.2	1.42	5.89	17.51

between the yields (88 % as against 17.4 %) in which the proteins had been prepared, the distributions of hexone bases are similar in both cases, confirming the view expressed above that these isolated proteins, although obtained from grasses in yields not exceeding 30 %, may be taken as representative of the whole protein of the leaf.

SUMMARY.

The proteins from a number of typical forage grasses (including red clover) have been analysed for the basic amino-acids by individual isolation of the latter. One large scale analysis of a cocksfoot protein showed the following yields (expressed as N in % of protein-N): arginine, 13·6; histidine, 1·66; lysine, 5·27. Small scale analyses of the proteins from other grasses gave: arginine, 12·1–13·1; histidine, 1·9–2·7; lysine, 4·4–5·3. A protein extract obtained from a sample of mixed pasture grass gave similar values.

A Van Slyke analysis of a cocksfoot protein indicated: arginine, 13·6; histidine, 7·1; lysine, 8·1. Reasons for these large figures are suggested and the method criticised.

Grateful acknowledgment is made to Prof. A. C. Chibnall for much help and advice. The cost of this research has been defrayed by a grant from Imperial Chemical Industries, Ltd.

REFERENCES.

- Block (1934). *J. Biol. Chem.* **106**, 457.
Chibnall, Miller, Hall and Westall (1933). *Biochem. J.* **27**, 1879.
Gortner and Sandstrom (1925). *J. Amer. Chem. Soc.* **47**, 1663.
Larmour (1928). *Trans. Roy. Soc. Canada* [iii], **22**, 349.
Miller and Chibnall (1932). *Biochem. J.* **26**, 392.
Morris and Wright (1933, 1). *J. Dairy Res.* **4**, 177.
—— — (1933, 2). *J. Dairy Res.* **5**, 1.
Osborne (1924). *The vegetable proteins.* (Longmans, Green and Co., London.)
Vickery and Leavenworth (1928). *J. Biol. Chem.* **76**, 707.
Vickery and Shore (1932). *Biochem. J.* **26**, 1101.