CLVI. THE BASIC AMINO-ACIDS OF LEAF PROTEINS. WITH A DISCUSSION OF VARIOUS METHODS OF ANALYSIS

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DURING the past few years numerous protein preparations have been made in this laboratory from forage plants at various stages of growth and growing under different climatic and manurial conditions. To compare these products one with another, and with the whole leaf protein of which they represent a part, it has been necessary to make amino-acid analyses not only of the protein preparations themselves, which may contain some 13–15 % N, but also of leaf residues containing unextracted protein or of leaf material containing the whole of the protein, both of which latter products, after removal of non-protein-N, are rich in carbohydrate and may contain only 3 or 4 % N. It has been thought advisable, therefore, to find out first of all whether the amino-acids set free during acid hydrolysis of the protein would condense with products derived from the carbohydrate and thus give low yields on subsequent analysis.

Miller [1935] has already carried out exploratory work on these lines with respect to the bases, and his conclusions can be summarized as follows.

(1) The large-scale method of Vickery and his co-workers [cf. Vickery & Shore, 1932] was the most reliable, but the amount of material required (50 g. or more) made its general use impracticable.

(2) Block's [1934, 2] small-scale adaptation of the above method, after certain modifications suggested by his (Miller's) experience, gave values which agreed closely with those obtained by the large-scale procedure.

(3) The N-distribution method of Van Slyke gave values with leaf proteins which could only be regarded as extravagant.

Miller also expressed the opinion that the destruction of bases which might occur during acid hydrolysis of leaf proteins in the presence of considerable amounts of carbohydrate impurities would not be very profound, even though the earlier work of Roxas [1916] had shown that small amounts of humin-N were formed when the free bases were heated with carbohydrates in acid solution. But the recent work in this laboratory of Bailey [1937] and of Lugg [see Chibnall, 1939, Appendix I] has made it clear that the impurities present in leaf products are those (e.g. mucilage, pentosan) which give rise to much furfuraldehyde on acid hydrolysis, and that under these conditions considerable destruction of certain of the amino-acids does indeed occur. Bailey, for instance, has shown that, in the case of cysteine, destruction or modification occurs more readily at the time of its actual liberation from the protein molecule than when it is present as the free amino-acid, while Lugg [1938, 1, 2] has pointed out the difficulties which attend the estimation of cystine, methionine, tyrosine and tryptophan in protein products containing such impurities. These findings have necessitated a re-examination of the methods of base analysis to ascertain what, if any, loss of each individual base occurs during hydrolysis under similar conditions.

As the amount of material available was in most cases limited the method chosen for investigation was that of Block [1934, 2], whose original procedure has been modified by him in recent years as his own extensive experience has suggested [cf. Block, 1938]. In the course of the present work it has likewise been found that certain suitable though minor changes can be recommended, and the procedure finally adopted for the analysis of leaf products, which incorporates suggestions made by Miller [1935] and by Ayre [1938], has been rigorously tested against appropriate mixtures of bases as well as against certain standard proteins which had already been analysed by the large-scale methods of Vickery and his co-workers. It will facilitate a critical discussion of the results obtained if the new procedure be first of all described in detail.

The modified method of base analysis

All concentrations are carried out *in vacuo* at 45° and all precipitates are washed three times unless otherwise stated.

Hydrolysis. 2.5–3.0 g. of protein are hydrolysed by boiling under reflux with a mixture of 5.5 ml. H_2SO_4 and 19.5 ml. water for 18–22 hr. (It is necessary to heat on the water bath until the protein has dissolved.) The hydrolysate is cooled, transferred to a 250 ml. pyrex centrifuge cup and treated with hot saturated baryta to pH 3 (indicator thymol blue). (Block [1934, 2] advised pH 3–6 but if the solution is made less acid than pH 3 the complete extraction of N from the BaSO_4 precipitate becomes difficult if the hydrolysis has given much acid humin.)

The precipitate is centrifuged, transferred to an end-runner mill (a heavy mortar and pestle will suffice) and ground with acidulated water (pH 3). The resulting cream is then poured into a beaker, boiled with vigorous stirring for 10 min., cooled and again centrifuged. The washing is twice repeated, about 400 ml. water being used each time. The solution and washings are concentrated to 20 ml. and transferred to a 250 ml. centrifuge cup, any slight precipitate of BaSO_4 being ignored.

Histidine fraction. Ag_2O and H_2SO_4 (sufficient to maintain the pH) are added until Ag_2SO_4 crystallizes in appreciable amount; AgNO_3 is then added to ensure excess Ag ion (brown precipitate with baryta). This variation of Block's procedure is recommended, as the exclusive use of AgNO_3 introduces nitrate ions in such concentration that partial destruction of lysine may occur at a later stage. The solution is next adjusted to pH 7.2–7.4 (blue-green with bromothymol blue) by the addition of cold saturated baryta; it is important that during this operation the stirring be efficient since a local concentration of baryta tends to precipitate arginine silver, which does not redissolve very readily above pH 4. If the end-point is exceeded (so that the solution is alkaline to phenolphthalein) the solution must be acidified to pH 4.0 (Congo red), stirred for several minutes and the adjustment of pH repeated. The precipitate of histidine silver is then centrifuged and washed twice with water (50–60 ml. in all), the clear liquid and washings (A) being acidified to pH 5 and treated for arginine as described below. The histidine precipitate is suspended in water acidified with H_2SO_4 to pH 4 (bromocresol green) and freed from silver by H_2S under pressure. The end-point is invariably marked by coagulation of the Ag_2S . The precipitate of Ag_2S and BaSO_4 is centrifuged, thoroughly washed and the collected liquors concentrated to 5 ml. The concentrate is transferred to a 100 ml. centrifuge bottle (final volume 15 ml.), 50 ml. of a saturated solution of HgSO_4 in 5% H_2SO_4 are added and the mixture placed in the ice box for at least 12 hr. (It is very convenient to leave histidine in this form until the arginine and lysine have been dealt with.)

The mercury compound is centrifuged, washed once with the reagent and decomposed with H_2S . The filtrate and washings are concentrated to 5 ml., neutralized with baryta, BaCO_3 added and the mixture brought to the boil. $\text{Cu}(\text{OH})_2$ or CuCO_3 is now added until in excess and the boiling maintained for 15 min. The mixture is cooled for at least 30 min. at 0° and the precipitate removed and washed. The filtrate is acidified until the solution becomes pale green, Cu is removed with H_2S and the CuS washed. The filtrate is now concentrated to 50 ml., an aliquot withdrawn for determination of total N and the remainder concentrated to 2 ml. Solid flavianic acid (15 mg. per mg. N) is added and the histidine diflavianate allowed to crystallize for 2-3 days at 0° . The diflavianate is filtered, washed with a little cold water containing flavianic acid, with ice-cold absolute alcohol, then with ether and finally dried at 105° . If the canary-yellow solid turns orange it must be purified by recrystallization. The S content should be 8.17%, but in keeping with the experience of Vickery and his colleagues the products isolated in this laboratory have generally given a slightly higher value, *circa* 8.38% (Schoeller, micro-Carius). The factor for converting the weight of diflavianate into histidine is 0.198.

Arginine fraction. The solution (A) is concentrated, in the presence of any precipitate which may have formed, to 25 ml., transferred to a 250 ml. centrifuge cup, tested for excess silver and the pH adjusted to 12-13 with a hot saturated solution of baryta (lanacyl blue or alizarin yellow). The precipitate is centrifuged, washed twice with cold saturated baryta and the total volume of liquid noted for subsequent correction for the solubility of arginine silver. The liquid (L) is treated later for lysine.

The precipitate is suspended in water, H_2SO_4 is added to pH 4, the silver removed with H_2S under pressure (coagulation is again a test) and the precipitate washed. The solution and washings are concentrated to 100 ml.; 1 ml. is withdrawn for determination of total N, the remainder adjusted to pH 5-6 (bromocresol green) and concentrated. Any precipitate of BaSO_4 is removed by filtering through a No. 42 Whatman filter paper and the final solution is concentrated to 2 ml. in a 150 ml. flask. The capillary and Claisen head of the flask are washed down to make a final volume of 10 ml.

The solution is heated on the water bath at 100° , the required amount of flavianic acid added in concentrated aqueous solution (6 mg. acid per mg. N) and the mixture shaken until crystallization commences. After standing overnight at 0° the flavianate is filtered, washed successively with cold water, alcohol and ether, dried at 105° and weighed. The S content should be 6.56%, but the products isolated in this laboratory have always given a slightly higher value, *circa* 6.72% (Schoeller, micro-Carius). The factor for converting arginine flavianate into arginine is 0.3566.

Lysine fraction. Use is made of the finding of Ayre [1938] that ammonium phosphotungstate is not decomposed by dilute H_2SO_4 in the presence of amyl alcohol-ether, thus eliminating the removal of NH_3 with NaOH .

The filtrate (L) is acidified to pH 6; 1.1 ml. conc. H_2SO_4 are added and excess silver removed as Ag_2S . The precipitate is well washed and the filtrate concentrated to 15 ml. Care should be taken not to reduce the volume below this owing to the presence of HNO_3 .

The solution is transferred to a centrifuge cup (volume now 30 ml.) and heated to 90° on a water bath for 3 min.; 50 ml. of a 20% solution of 24-phosphotungstic acid (A.R.) in 5% H_2SO_4 , also at 90° , are added and the phosphotungstate allowed to crystallize overnight at 15- 20° . The precipitate is washed twice with a solution of 2.5% phosphotungstic acid in 5% H_2SO_4 . The phosphotungstates are

then decomposed by disintegrating with 50 ml. of water (to which has been added 1 ml. H_2SO_4) and a liberal quantity of amyl alcohol-ether (25 parts ether, 20 parts amyl alcohol, 1 part ethyl alcohol) and the mixture centrifuged. The clear liquids are poured into a separating funnel. The residue, which consists of ammonium phosphotungstate, is washed twice, as before, using smaller quantities of acidified water and organic solvents, the washings being added to the mixed liquids in the separating funnel. After allowing the solutions to clear, the aqueous solution is drawn off and shaken out three times successively with amyl alcohol-ether. The combined organic solutions are shaken once with a small quantity of acidified water, and the latter solution washed once with amyl alcohol-ether. The aqueous solutions are then combined and given a final washing with a small quantity of amyl alcohol-ether.

This aqueous solution is then concentrated to 100 ml.; aliquots are withdrawn for determination of total N and the remainder treated for the separation of lysine picrate. The solution is adjusted to pH 6.5–7.0 (bromothymol blue) with baryta; 1.0 g. BaCO_3 is added, the precipitate is centrifuged and well washed. The filtrate is then concentrated in the presence of BaCO_3 (1.0 g.) and filtered through a No. 42 filter paper. The final solution is concentrated to 1 ml., the capillary and flask head washed down with 6 ml. 93% alcohol, the resulting liquid is adjusted with absolute alcohol or water so that a faint turbidity remains and is then cooled to 0° . An alcoholic solution of picric acid (8.1 mg. acid per mg. N), cooled to 0° , is then added. (It is necessary to cool to 0° because the use of a warm alcoholic solution of picric acid may lead to the formation of an oil.)

The picrate is allowed to crystallize overnight at 0° , washed with ice-cold alcohol and ether, dried at 105° and weighed (in case loss occurs during recrystallization); it is recrystallized from water at 0° (solubility 3.4 mg. per ml. at 0°), dried as before and weighed. It has been the unanimous experience of those who have used the original Block or the above-mentioned modified procedure in this laboratory that the lysine picrate, prepared by using either A.R. picric acid or the product purified according to the directions of Block [1938], will not explode above 260° unless recrystallized. The factor for the conversion of the weight of lysine picrate into lysine is 0.39.

Corrections for solubilities and overall losses in procedure

An arginine silver correction of 1.1 mg. N per 100 ml. [Gulewitsch, 1899] has been applied by Vickery & Shore [1932] and by Vickery & White [1933] in their large-scale analyses of ovalbumin and casein respectively. A perusal of Block's earlier publications suggests that he also, in certain instances, has applied this correction, but no specific reference to it is made in the paper [1934, 2] which describes his original procedure, while in his later review [1938] he merely quotes, in a special table, without further comment, the solubility value, together with those of other base salts. It is to be remembered, of course, that the solubility of a salt such as arginine silver in any given solution will be governed by the amounts of other amino-acids or their salts present [cf. Cohn, 1935], a point that is strikingly illustrated by the data concerning the solubility of arginine phosphotungstate discussed in a later section; consequently the employment of such a correction in the case of protein hydrolysates is somewhat arbitrary and opportunity has been taken to investigate the matter, in admittedly simpler systems, as part of an enquiry into the losses inherent in the above-mentioned procedure for separating the three bases.

For this purpose arginine and histidine were prepared from their recrystallized flavianates and lysine from its recrystallized picrate, the amount of each

base taken in the experiments about to be described being equivalent to the respective occurrence in 2.5 g. of a typical leaf protein. In exp. 1 arginine alone was used, silver being precipitated at pH 7 as though histidine were present. 1.1 mg. of N were lost in this "histidine precipitate". After precipitation of the arginine silver at pH 14 the mother liquor contained 3.3 mg. of N, equivalent to a solubility of 2.0 mg. per 100 ml. Two other experiments (unrecorded) gave similar results. In exps. 2 and 3, with histidine also present, the histidine silver precipitates contained 2.2 and 1.2 mg. of N respectively in excess of the histidine-N present in the original solution, while the arginine silver mother liquors contained 1.4 mg. and 3.3 mg. of N, equivalent to solubilities of 1.0 mg. and 1.4 mg. per 100 ml. respectively. In exps. 4 and 5, containing all three of

Table I. *Analysis of various mixtures of bases*

	Arginine-N					
	Taken mg.	Recovered mg.	Loss mg.	Corrected for arginine silver 1.1 mg. N per 100 ml.		
				Recovered		Loss mg.
				mg.	%	
1	37.9	33.4	4.5	36.1	95.2	1.8
2	37.9	34.3	3.6	35.9	94.7	2.0
3	37.9	33.4	4.5	35.2	92.9	2.7
4	38.2	33.7	4.5	35.9	94.0	2.3
5	38.2	33.1	5.1	35.3	92.4	2.9
6	42.8	38.8	4.0	40.5	94.5	2.3

	Histidine-N				Lysine-N			
	Taken mg.	Recovered		Loss mg.	Taken mg.	Recovered		Loss mg.
		mg.	%			mg.	%	
1	—	—	—	—	—	—	—	—
2	9.0	—	—	—	—	—	—	—
3	9.0	—	—	—	—	—	—	—
4	6.4	6.2	96.8	0.2	18.4	16.9	91.8	1.5
5	6.4	6.0	93.8	0.4	18.4	17.6	95.5	0.8
6	10.2	10.0	98.5	0.2	25.9	24.1	93.0	1.8

the bases, the arginine silver mother liquors contained 3.0 mg. and 3.6 mg. in excess of the known content of lysine-N, equivalent to solubilities of 1.5 mg. and 1.8 mg. per 100 ml. respectively. Exp. 6 was similar to 4 and 5, except that the mixture was submitted to the usual acid hydrolysis before separation, the solubility of the arginine silver in this case being 1.6 mg. per 100 ml. In all these experiments, as in the protein analyses quoted later, there was no loss on recrystallization of the arginine flavianate. These results show that under the working conditions of these small-scale experiments the apparent solubility of arginine silver may be a little greater than that recorded by Gulewitsch [1899], and that it is not appreciably increased in the presence of lysine. Assuming for convenience, therefore, that the Gulewitsch correction (1.1 mg. N per 100 ml.) holds, the overall losses in the estimation of arginine, of mean value 2.6 mg. N, are to be ascribed to possible underestimation of this solubility factor and to precipitation with the histidine silver at pH 7. Whether such an overall correction is valid in the case of a protein hydrolysate must remain a matter of conjecture until new methods of analysis are found which permit a direct

determination of solubility under such conditions [cf. Bergmann & Stein, 1939] and it is only with this reservation that the correction has been applied in the analyses quoted later in Tables II and V.

The estimation of N in the histidine silver fraction of expts. 2-7 confirms the findings of Vickery that this salt is completely insoluble and that the mean overall loss of 0.3 mg. is due to the difficulty of isolating such small amounts of histidine diflavinate. With the reservation given above, correction for this small overall loss has been applied in the analyses quoted later in Tables II and V. In this connexion certain inconsistencies in Block's publications must be noted. A histidine value of 0.91 % for eight mammalian serum proteins quoted by Block *et al.* [1934] was corrected to 2.1 % by Block [1934, 1] for "the average loss of histidine incident to the method of estimation", but in his later review [1938] he states that the "recovery of histidine as diflavinate is approximately 75 % of that present". Such statements are entirely unsupported by any published experimental evidence and in view of the results quoted in Tables I and III of the present paper they appear to be unjustified.

The average overall loss of 1.6 mg. of lysine-N in expts. 4-11 (Tables I and III) covers the solubility of lysine phosphotungstate (volume about 175 ml.) and all the incidental losses of procedure. As will be discussed later, there is clear evidence that in certain protein hydrolysates the solubility of arginine phosphotungstate is no greater than that determined by Van Slyke in an equivalent mineral acid solution, while in others this value is greatly exceeded, so that in the case of the protein analyses quoted below the true overall loss of lysine-N may well be greater (or less) than the value found for the simple base mixture. This reservation, of course, applies with equal force to the large-scale work of Vickery and co-workers and also to the Van Slyke method of base analysis, consequently it cannot be regarded as one of the contributory causes of the high values invariably obtained for lysine by the latter procedure. To clarify the situation we must again await the development of methods for the estimation of this base in which the residual solubility can be directly computed; meanwhile an overall correction of 1.6 mg. of lysine-N has been applied in the protein analyses given below.

Analysis of certain standard proteins

The ovalbumin and casein I were kindly supplied by Dr Vickery, being samples of the actual preparations analysed by Vickery & Shore [1932] and Vickery & White [1933] respectively; the edestin and the other samples of casein were prepared in this laboratory and contained 18.6, 14.83 and 15.7 % N respectively. The results of analysis, together with those of other workers for comparison, are collected in Table II.

It will be seen that the values for the individual bases, when calculated simply on the weights of crystalline derivatives, tend, in the small-scale procedures, to be lower than those obtained by working on a larger scale. This is to be expected, since in the latter case the mother liquors from the separation of the main crop of derivate can be subsequently worked over to increase the yield. As has been shown earlier, this drawback to the use of the small-scale procedure can, with certain assumptions, be overcome by the use of overall corrections for losses, and when these are applied the agreement between the two sets of results is as close as one could reasonably expect.

Table II. *Base analysis of standard proteins*

(Values quoted are percentages of total protein-N)

Protein	Analysed by	Wt. of protein used g.	Arginine-N		Histidine-N		Lysine-N	
			Found (corrected for arginine silver 1.1 mg. N per 100 ml.)	Corrected for overall losses (+ 2.6 mg. N)	Found	Corrected for overall losses (+ 0.3 mg. N)	Found	Corrected for overall losses (+ 1.6 mg. N)
Ovalbumin	Author	2.89	11.05	11.65	2.55	2.63	5.5	5.85
Ovalbumin	Author	2.52	11.6	12.3	(Lost)	—	5.6	6.00
Ovalbumin	Vickery & Shore [1932]	65.0	11.6	—	2.1	—	5.6	—
Ovalbumin	Vickery & Shore [1932]	65.0	10.7	—	2.6	—	6.1	—
Casein I	Author	2.7	7.7	8.3	3.02	3.1	7.4	7.8
Casein II	Author	2.3	7.5	7.8	—	—	—	—
Casein III	Author	2.61	7.8	8.4	3.2	3.3	7.45	7.85
Casein I	Vickery & White [1933]	100	7.7	—	—	—	—	—
Casein I	Vickery & White [1933]	100	8.2	—	3.2	—	7.72	—
Edestin	Author	2.56	26.4	27.0	3.02	3.08	2.25	2.59
Edestin	Author	2.46	26.2	26.7	2.84	2.89	2.00	2.35
Edestin	Vickery & Leavenworth [1928]	366	27.4	—	3.04	—	2.26	—
Edestin	Vickery & Leavenworth [1928]	50	23.8	—	2.6	—	2.04	—
	Block [1934, 2]	2.5	27.05*	—	3.06	—	2.17	—

* No mention is made of any correction for arginine silver.

General criticism of methods of base analysis

From the experience gained in the development of the present research the following criticisms may be levelled against the small-scale gravimetric method of analysis. (1) The use of AgNO_3 , even in the limited amounts suggested in the procedure detailed above, may cause loss of base, particularly of lysine. It was found impossible, however, under the conditions of these experiments, to introduce an excess of Ag ions solely by means of Ag_2O or Ag_2SO_4 . (2) If the lysine fraction is relatively impure, and this occurred occasionally in the analyses of leaf proteins, the lysine dipicrate may separate in the form of an oil from which it was found impossible to regenerate the base quantitatively for reprecipitation. Repetition of the whole base analysis was therefore necessary in such cases. (3) The estimation of histidine is the least satisfactory of the three bases, as Vickery and his co-workers found when working on a large scale. This is due in part to the small amount of the amino-acid present in most protein hydrolysates, and in part to the difficulty of obtaining the diflavinate in a reasonable state of purity. If, for instance, a large excess of flavianic acid be added to prevent the formation of a monoflavinate, the excess acid may crystallize out and as this must subsequently be washed away with cold water a loss of diflavinate may result. It was presumably to overcome some of these difficulties that Block has recently suggested the use of nitranilic acid as a precipitant for this base, but he has not as yet given any data for the recovery of histidine nitranilate from pure base solutions or from protein hydrolysates. Finally, from what has been said above concerning the possible vicarious

solubilities of arginine silver and lysine phosphotungstate, it may well be that both the large- and small-scale gravimetric methods are still underestimating the content of one or more of the bases in certain proteins.

This latter statement in no way implies, however, that the higher values usually obtained for histidine and lysine by the indirect Van Slyke method of base analysis are valid. Much has been written in the past as to the trustworthiness of this method, and some of the criticisms put forward have been recently summarized by Calvery [1938], who points out that the procedure has frequently been used in a way not intended by Van Slyke, namely, as an absolute rather than as a comparative method of analysis. The main objections are: (1) The phosphotungstate precipitate rarely, if ever, contains exclusively bases; small amounts of tyrosine, tryptophan and phenylalanine have been isolated from it in certain cases while Gortner & Sandström [1925] have pointed out the possible interference of proline. (2) If large amounts of cystine are present in the protein hydrolysates the determination of arginine is affected, since concentrated NaOH converts up to 20 % of the cystine-N into NH_3 . (3) Enhanced values for arginine-N and amino-N are reflected in the calculated values for lysine-N and histidine-N.

Another important observation has, however, been overlooked by Calvery, namely, that the solubility of arginine phosphotungstate in a protein hydrolysate may be greater than the value (about 1.6 mg. N per 100 ml.) recorded by Van Slyke. This was first pointed out by Vickery & Leavenworth [1928], who were able to isolate from the phosphotungstate filtrate of edestin, *via* the silver salt, more than twice the expected amount of arginine flavianate. In the course of the present work a similar result was given by myosin, yet in the case of casein, and also of many leaf proteins, the Van Slyke solubility factor was valid, since the amount of arginine flavianate obtained from the phosphotungstate precipitate, when given this correction, was equal to that obtained from the hydrolysate direct. Clearly the solubility of arginine phosphotungstate depends on the amino-acid mixture present in the protein hydrolysate [cf. Cohn, 1935] and the fact that this may be in excess of the Van Slyke value lends added weight to the remarks which follow.

Miller [1935], working in this laboratory, applied both the gravimetric and indirect methods of analysis to cocksfoot leaf protein, and found that the total N precipitated by phosphotungstic acid under the Van Slyke conditions was greatly in excess of the total basic N determined gravimetrically by the large-scale procedure of Vickery, the latter value being more or less equivalent to the total N precipitated by phosphotungstic acid under the Hausmann conditions. Since in the Van Slyke procedure the precipitation with phosphotungstic acid is carried out in the presence of HCl—instead of H_2SO_4 as in the Hausmann procedure—he suggested that the high value obtained in the former case might be due to precipitation of certain non-basic substances under such conditions. Further consideration, however, does not lend support to such a view, for these differences can be accounted for in large part in terms of the solubility of arginine phosphotungstate, as recorded by Van Slyke, and of the different volumes of solution from which the precipitations are made in the two cases. Indeed such relatively large volumes of solution are employed in the Hausmann procedure that the phosphotungstate filtrate and washings must contain much arginine-N, and the general agreement between the Hausmann basic-N and the gravimetric total basic N, which was stressed by Osborne [1924, p. 73], must be largely fortuitous and due to the presence of non-basic products in the Hausmann as well as in the Van Slyke precipitate. In view of all these criticisms it is

impossible to agree with the conclusion of Calvery [1938] that "in comparing the Van Slyke method with that of Kossel (i.e. Vickery, Block), it is generally true that in the case of lysine the Van Slyke method is more accurate".

Analysis of a mixture of the three bases after hydrolysis in the presence of various carbohydrates

Before applying the above-mentioned gravimetric procedure to the analysis of leaf protein preparations it was necessary to ascertain the effect, if any, which the carbohydrates known to be associated with the proteins in these products would have on the recovery of the three individual bases. Appropriate mixtures of the bases (representing 2.5 g. of a leaf protein) were accordingly hydrolysed in the presence of various carbohydrates which yield much furfuraldehyde under such conditions, and the subsequent analyses are given in Table III, the value for recovered arginine-N carrying a silver correction of 1.1 mg. per 100 ml. In agreement with the previous finding of Roxas [1916] arginine has suffered a loss which is roughly proportional to the amount of carbohydrate present, but the change undergone by the amino-acid has not been ascertained, save that no NH_3 is produced and that the humin-N formed accounts for only a part of the lost arginine-N. Histidine recoveries are irregular, and, except in the case of exp. 15, are probably associated with the difficulties of estimation (see earlier) rather than with destruction of the amino-acid. Lysine recoveries in exps. 8-14 are normal, but the small loss shown in exp. 15 may be significant, since it will be shown in a later paper that much destruction of this amino-acid can occur if the amount of impurity be increased to the equivalent of 10 times the protein present. That destruction of base, particularly of arginine, may be more extensive at the time of liberation either as peptide or as free amino-acid during hydrolysis of a protein [cf. Bailey, 1937] follows from the results of exps. 12 and 13, although in these particular cases the total amount of arginine concerned was very much greater than in the others, wherein the arginine content was designedly made more or less equivalent to that of a typical leaf protein, and comparison may not be valid. In view of these relatively large losses of arginine in the presence of much carbohydrate and the uncertainty in the recovery of histidine it has been thought advisable in dealing with what can only be regarded as grossly impure protein preparations, e.g. those with 8% N or less (see section on whole grass analyses) to limit the base analysis to the determination of lysine.

Table III. *Recovery of bases after hydrolysis in the presence of various carbohydrates*

Exp.	Added carbohydrate	Wt. g.	Arginine-N				Histidine-N				Lysine-N			
			Taken mg.	Recovered			Taken mg.	Recovered			Taken mg.	Recovered		
				mg.	%			mg.	%			mg.	%	
8	—	—	42.8	40.4	94.5		10.2	10.05	98.5		25.9	24.1	93	
9	Arabinose	0.25	42.8	37.0	86.5		10.2	8.1	79.5		25.9	24.6	95	
10	Arabinose (a)	0.25	42.8	36.6	85.5		10.2	9.9	97		25.9	23.9	92.4	
11	Arabinose	0.5	52.0	43.7	84.0		7.1	7.1	100		28.4	26.4	93	
12	Arabinose (b)	0.5	136.3	114.5	84.0		15.1	14.1	93.5		11.3	10.8	96	
13	None (b)	—	136.3	131.0	96.0		15.1	14.6	96.8		11.3	10.6	93.9	
14	Xylose	0.3	52.0	45.5	87.6		7.1	7.02	98.7		28.4	27.0	95	
15	Hemicellulose	2.5	52.0	37.8	72.7		7.1	4.9	69		28.4	25.5	90	

(a) 0.1 g. cystine and 0.1 g. tryptophan were added to this mixture before hydrolysis.

(b) 2.68 g. edestin instead of mixed bases were used in this experiment. The amount of each base was calculated from the data of Table II, i.e. arginine-N = 27.4%; histidine-N = 3.04%; lysine-N = 2.26%.

Base analysis of various leaf protein preparations

The samples of forage proteins used had been prepared by Chibnall and his colleagues in past years by the "used ether-water" method and most of them have been recently analysed by Lugg [1938, 1, 2] for amide-N, tyrosine-N, tryptophan-N, cystine-N and methionine-N. For continuity the series numbers quoted by Lugg have been retained in Table V. The samples of maize and castor bean proteins were those prepared by Chibnall, using the "ether" method [cf. Chibnall, 1924; Chibnall & Nolan, 1924]. All these products contained 12.5–15.7 % N and since there is reason to believe that pure leaf proteins should contain about 17 % N [cf. Chibnall, 1939] it has been assumed that the adulterant—of the pentosan nature referred to above—will cause destruction of arginine during acid hydrolysis in amount proportional to that of the adulterant present (cf. Table III).

Table IV. *Factors used for correcting values of arginine-N in leaf protein preparations*

% N	17.0	16.0	15.0	14.5	12.5–13.0
Calculated % adulterant	0	5.9	11.8	14.7	20.0–30.0
Factor for loss	1.06	1.10	1.15	1.17	1.20

Table V. *Base analysis of leaf protein preparations*

(Figures given are in percentages of total protein-N)

Preparation no.	Source	Date	Total N (ash-free) %	Arginine-N	Histidine-N	Lysine-N
4	<i>Dactylis glomerata</i> (cocksfoot grass)	29. ix. 36	14.25	13.9	2.3	6.3; 6.1
2E(b)	"	7. vi. 37	12.76	15.5; 15.2	2.4; 2.6	6.0
16	<i>Lolium Italicum</i> (Italian rye-grass)	26. vi. 33	14.1	13.4	2.4	5.9
14	<i>Lolium perenne</i> (perennial rye-grass)	6. xi. 33	13.0	14.5	2.4	6.2
3E(a)	"	9. vi. 37	13.75	13.4	—	5.8
18	<i>Poa trivialis</i> (rough-stalked meadow grass)	27. vi. 33	14.1	16.4	2.3	5.6
—	"	4. x. 32	13.6	15.3	2.1	5.4
20	<i>Festuca rubra</i> var. <i>Fallax</i> (chewings fescue)	29. vi. 33	14.7	14.3	1.9	5.2
19	"	26. ix. 32	14.4	14.6	2.0	5.7
22	<i>Cynosurus cristatus</i> (crested dog's tail)	12. vi. 33	14.3	15.1	2.6	5.8
25	<i>Medicago sativa</i> (lucerne)	29. vi. 33	14.4	15.1	2.3	7.0
26	<i>Trifolium repens</i> (wild white clover)	18. x. 33	13.3	15.4	1.6	6.6
27	<i>Trifolium pratense</i> (red clover)	23. x. 33	12.95	14.9	2.3	6.5
32	<i>Spinacea oleracea</i> (spinach)	18. vi. 24	15.4	14.1	2.2	6.2
—	<i>Beta cicla</i> (beet spinach)	1. xi. 37	15.0	12.4	2.1	6.8
5E1	"	3. xii. 37	14.2	13.1	2.4	6.6
—	<i>Zea Mays</i> (maize)	1924	13.95	14.4	2.1	6.1
—	<i>Ricinus communis</i> (castor bean)	1924	12.55	12.9	2.2	6.5

The factors used in correcting for this loss are given in Table IV, and in the light of the previous discussion of expts. 12 and 13 they can only be regarded as minimal, a point that must be remembered in comparing the values for arginine-N given in Table V. Factors of 1.07 and 1.03 for lysine-N and histidine-N respectively have also been applied, irrespective of the amount of adulterant present.

The base analyses, like those of Lugg for the other amino-acids and the amide-N, exhibit surprisingly little variation from species to species, showing that leaf proteins, as a class, are of much more uniform composition than, for instance, a typical class of seed proteins such as the globulins [cf. Osborne, 1924]. The importance of this observation in problems connected with agricultural nutrition has already been discussed at some length by Chibnall [1939].

Base analysis of the whole protein of leaves

The extraction of protein material from leaves by the methods used in preparing the samples mentioned in Table V depends on the efficacy of the grinding operations employed, and the debris of leaf material may contain 70–85 % of the total amount of protein originally present in the untreated leaves. From a nutritive point of view the composition of this unextracted protein is of equal importance to that of the isolated product, but the difficulties attending its analysis are great, on account of the large amount of non-protein material with which it is associated. These leaf residues, or, in certain cases, the fresh leaves containing the whole of the protein, were prepared for analysis in the following way.

The dried and ground leaf material was first treated at boiling point with 0.01 *M* acetic acid to remove all soluble non-protein nitrogenous products, sugars etc., then successively with graded strengths of boiling alcohol to remove most of the lipin, with boiling 0.005 *M* citric acid (which usually reduces the ultimate ash content but removes only a negligible amount of nitrogenous material), twice with boiling alcohol (or until extract was almost free from chlorophyll) and finally with warm ether. Such residues, regarded as proteins, are grossly impure, since they will contain only from 2 % N to (rarely) 8 % N. In one particular case the dried grass contained 4.2 % N, and after treatment in this way the leaf residue contained 5.6 % N. If this N represents protein, and this is assumed, then, from the point of view of hydrolysis, the impurity present was about 200 % and to reduce this the leaf residue was boiled for 24 hr. with 7.5 % H_2SO_4 in amount such that, on filtration and concentration of the filtrate, the correct conditions for a base analysis could be achieved. About 92 % of the residual leaf-N was extracted in this way and, by difference, the extracted material contained 7.9 %, so that the subsequent hydrolysis took place in the presence of 100 % impurity. Reference to Table III shows that both arginine and histidine may suffer considerable destruction under such conditions and lysine was regarded as the only base which could be estimated with any reliability, the recovery being taken as 90 %. In another case the residual leaf material already contained 8 % N, and opportunity was taken to compare the lysine values both with and without this preliminary extraction with dilute H_2SO_4 .

The results, together with those of corresponding protein preparations, are given in Table VI, and they show that within the limits to which we can work at the present time the values for lysine, like those for amide-N and the other amino-acids recorded by Lugg [1939; cf. Chibnall, 1939] exhibit but little

Table VI. *Lysine analyses of various protein fractions of leaves*

Preparation no.	Source	Date	Season	Material*	% of total leaf protein represented	Total N %	Lysine-N % of total protein-N
2Eb	<i>Dactylis glomerata</i> (cocksfoot)	7. vi. 37	Spring	E	25	12.8	6.0
—	"	5. vi. 32	"	W	90	8.0†	5.9
—	"	5. vi. 32	"	W	100	8.0†	6.1
—	"	29. ix. 36	Autumn	E	39.4	14.2	6.2
1W	"	29. ix. 36	"	W	91	7.5†	5.9
3E (a)	<i>Lolium perenne</i> (perennial rye-grass)	9. vi. 37	Spring	E	24	13.8	5.8
—	"	6. xi. 33	Autumn	E	21.8	13.0	6.2
4W	"	29. xi. 37	"	W	89.3	5.6†	5.9
4R	"	29. xi. 37	"	R	56	3.4†	5.6
—	<i>Beta cicla</i> (beet spinach)	6. xi. 37	"	E	—	15.1	6.8
—	"	6. xi. 37	"	W	92.5	6.7†	6.7
5R	"	3. xii. 37	"	R	52	4.1†	6.6
5E	"	3. xii. 37	"	E	—	14.2	6.6

* Extracted protein (E); protein of leaf residues (R); whole protein of leaves (W).

† N content of leaf material before preliminary extraction with dilute H_2SO_4 .

‡ No preliminary extraction with dilute H_2SO_4 .

variation as between isolated protein, extracted residue and whole protein of leaf. Moreover, there is but little variation within a single species with season. The implications of these findings have been discussed in some detail by Lugg and Chibnall in the communications cited above.

SUMMARY

Block's [1934] small-scale adaptation of the Vickery method for the estimation of the basic amino-acids of proteins has been investigated in detail. Certain minor modifications are recommended, and the modified procedure has been tested against appropriate mixtures of bases to obtain some idea of the corrections to be applied for arginine silver and overall losses.

Various standard proteins have been analysed by this modified procedure and, after correction, the results compare favourably with those obtained by Vickery and his co-workers.

A general criticism has been made of certain direct and indirect methods of base analysis.

Hydrolysis of the three individual bases in the presence of various furfuraldehyde-yielding carbohydrates has shown that arginine suffers a loss which is roughly proportional to the amount of carbohydrate present, that histidine recoveries may be irregular and that lysine recoveries are normal unless a large excess of carbohydrate is present.

In the light of these results it has been possible to obtain fairly satisfactory analyses for all three bases of leaf protein preparations containing from 12 to 16% N.

Leaf material relatively poor in protein (containing less than 8% protein-N) requires special preliminary treatment, and it is considered that the only base which can be estimated with any reliability is lysine.

In keeping with the previous analyses for certain other amino-acids and the amide-N by Lugg, the base analyses of leaf proteins exhibit but little variation from species to species and within a single species with season.

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