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Partial Amino-acid Compositions of some Plant-leaf Protein Preparations: the Arginine, Histidine and Lysine Contents

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In an earlier publication (Lugg & Weller, 1944) the amide, tyrosine, tryptophan, cyst(e)ine (i.e. cystine plus cysteine calculated as cystine) and methionine contents of some extracted plant-leaf protein preparations were reported. These preparations, one each from leaves of *Phalaris tuberosa* L., *Hordeum murinum* L., *Medicago sativa* L., and *M. denticulata* Willd., were judged, both from the mode of preparation and from analytical evidence, to be reasonably representative of the whole proteins in the leaf materials. The arginine, histidine and lysine contents of these same preparations have now been estimated and it is one purpose of this article to record the values obtained. The question of the existence of hydroxylysine in these preparations has also been considered.

EXPERIMENTAL

Methods. The arginine and lysine contents of the preparations were estimated by methods based essentially upon those of Tristram (1939). Histidine contents were at first estimated by Tristram's (1939) method, but for reasons to be discussed, the resulting values were considered unsatisfactory and recourse was had to an adaptation of the method of Vickery & Winternitz (1944). A modification of the Kapeller-Adler (1934) colorimetric method of estimating histidine, recommended by Block (1937), was unsatisfactory in our hands.

Hydrolysis. In Tristram's experience the hydrolysis of plant-leaf protein preparations for the purpose of estimating the contents of these three amino-acids could be performed satisfactorily by heating the protein with about ten times its weight of 4M-H₂SO₄ for 18–22 hr. at the boiling point of

the mixture (approx. 110°). Whilst it is unlikely that these conditions would secure the maximum 'net production of amide NH₂ plus amino-acid "amino" groups' (see Lugg, 1946), they may achieve almost complete liberation of these particular amino-acids from such proteins. In our work, about 2.7 g. of the air-dried preparation (about 2.5 g. dry wt.) were heated for c. 1 hr. with 25 ml. of 4M-H₂SO₄ at 100° and then at the boiling point of the mixture for 21–22 hr., which is the upper region of Tristram's (1939) range.

Estimation of arginine, histidine and lysine. The excessive variability of replicate histidine values estimated by Tristram's (1939) method and the extreme uncertainty of values obtained by the Conrad & Berg (1937) modification of Kapeller-Adler's (1934) method (tints of standard and test colour solutions frequently differed markedly) made it desirable to find a more satisfactory procedure. The Vickery & Winternitz (1944) method, in which the histidine is estimated gravimetrically as the di-3:4-dichlorobenzene sulphonate (histidine-DBS), was adapted for use in a general procedure for the estimation of arginine and histidine.

In this procedure insufficient Ag₂O and H₂SO₄ for precipitation of Ag₂SO₄ to occur were used, and larger quantities of AgNO₃ than are needed in Tristram's (1939) procedure, had to be employed. This and subsequent steps were taken to prevent the introduction of variable amounts of H₂SO₄ and/or HCl, in the presence of which we found the solubility of histidine-DBS to be appreciably increased. Urea was added in small quantities to decompose any nitrous acid which may have been present (from decomposition of nitrate by reducing substances in acid solutions). Tristram's procedure was then followed for the separation of the arginine- and histidine-silver compounds and for the isolation of the arginine and lysine as monoflavinate and monopicrate, respectively.

The histidine was liberated from the silver compound by treatment with H₂S (Vickery & Winternitz, 1944, used HCl) and was then precipitated as histidine-DBS in the

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manner described by these authors, but from a volume of 10 ml. and with 1.5 g. of the acid. The salt was recrystallized repeatedly from aqueous solutions of the acid until the recrystallization loss was the same as that found for the pure salt. To the final weight were added the recrystallization losses of the pure salt for every recrystallization. Purity was confirmed by melting-point determination. A correction for overall losses of +2.1 mg. of histidine N was made (see later references).

The arginine monoflavinate was recrystallized repeatedly from water and the final weight of pure salt was corrected for the recrystallization losses. A correction for overall losses of +0.4 mg. N was made (see later references) in addition to the correction for solubility of the arginine-silver compound. This correction, which is much smaller than Tristram's, was applied also to results obtained by his unmodified method. Incidentally, Chibnall, Rees & Williams (1943) believed the earlier correction to be too high. Weights of crude salt obtained by Tristram's unmodified method were corrected for impurity by multiplying by a factor, 0.965, derived by recrystallizing samples obtained in several analyses and pooled samples from the other analyses.

The lysine monopicrate was tested for purity by fractional recrystallization of mixed samples and chromatographing the fractions; a general correction was then applied by multiplying the weights of crude material by 0.975. Tristram's (1939) correction for overall losses (+1.6 mg. N) was made.

Arginine, histidine and lysine were thus estimated by Tristram's (1939) methods (method 1), but histidine (and, in some instances, arginine) were estimated also by the procedures outlined above (method 2).

Losses of arginine, histidine and lysine in the analytical procedures. In the examination of losses, mixtures of the following quantities of amino-acids were used: approx. 0.12 g. L-arginine, 0.04 g. L-histidine, 0.12 g. L-lysine, 1.0 g. glycine, 0.5 g. DL-alanine and 0.5 g. DL-aspartic acid. These mixtures, dissolved in 25 ml. of 4M-H₂SO₄ solution, were (a) submitted to direct analysis, (b) 'hydrolyzed' at the boiling point for 21-22 hr. and then analyzed, (c) treated with 0.5 g. of L-arabinose before 'hydrolysis' and analysis.

Table 1. *Percentage losses of arginine (0.12 g.) and histidine (0.04 g.), when submitted with lysine (0.12 g.), glycine (1.0 g.), alanine (0.5 g.) and aspartic acid (0.5 g.) to the hydrolysis procedure in the absence and in the presence of arabinose*

Arabinose present (g.)	Losses (%)	
	Arginine	Histidine
0	0	0
0.50	16	4

From replicate (a) experiments were found the mean corrections for overall losses (previously given) for use with method 2. From replicate (b) and (c) experiments were derived the data shown in Table 1. In analytical procedures (such as these) which involve the separation of amino-acid salts of moderate solubility, losses might be expected to arise from any racemization suffered by the amino-acids. The results shown in Table 1, and those of Tristram (1939) for lysine, indicate that to whatever extent racemization might have occurred, recoveries of the amino-

acids could not have been affected by it. Losses in absence of carbohydrate, estimated by other methods also, appear to be zero for all three amino-acids concerned (see Chibnall, 1946).

Tests with regard to the possible presence of hydroxylysine in the protein preparations

Van Slyke, Hiller, Dillon & MacFadyen (1938) have described the isolation of lysine and hydroxylysine picrates from gelatin hydrolysates by a procedure which differs very little from that used in this work (above) for the isolation of lysine monopicrate, the main points of difference being that they repeatedly recrystallized the phosphotungstates to remove other amino-acids, and fractionally precipitated the picrates. They state that hydroxylysine picrate is rather more soluble than the lysine salt in water. From their work it may be presumed that, if the leaf-protein preparations were to contain appreciable quantities of hydroxylysine, its picrate would contaminate the crude lysine salt isolated in this work.

Samples of crude lysine monopicrate obtained in analyses of the four protein preparations were mixed in approximately equal amounts and submitted to fractional recrystallization from aqueous solution. A disgusting odour, suggestive of mustard and CCl₄, was evolved when the mixture was dissolved in hot water. By repeated fractional recrystallization five fractions were obtained. The head fraction accounted for 85.1% of the weight of the mixture, successive fractions for 1.6, 3.4 and 3.3, and the tail fraction 6.1%; 0.5% was not recovered, and may have been lost in the effluvia referred to above.

The five fractions were submitted to so-called 'one-dimension' partition-chromatographic analysis (Consden, Gordon & Martin, 1944). The stationary phase was phenol-saturated water containing 0.3% (w/v) of NH₃, and the mobile phase was water-saturated phenol. Preliminary liberation and separation of the amino-acids from the picric acid was found to be unnecessary, a picrate (NH₄ salt?) band travelling independently of the amino-acids. From the intensities of the band colorations it was estimated roughly that the amino-acids in the original mixture of crude picrates probably contained 98% of lysine. Of the remainder, only a small fraction, if any, could have been hydroxylysine.

RESULTS

The results of the analytical work on the protein preparations are given in Tables 2 and 3. In Table 2 are shown the percentages of the leaf-protein nitrogen found in the form of arginine, histidine and lysine in the hydrolysates with assessments of the random errors of estimation. The table is self-explanatory.

Evaluation of the analyses

It seems likely that method 2 would have given, if applied generally, arginine values similar to those obtained by method 1, but with considerably smaller variability of replicate estimations (to judge from the work with the test mixtures). The relatively large variability of replicate arginine estimations by method 1 is attributable, at least in part, to the fact that, although the degree of purity of the isolated crude arginine monoflavinate must have varied,

Table 2. Percentages* of protein N found in the hydrolysates in the forms of arginine, histidine and lysine (corrected only for systematic errors of analysis of hydrolysates), and other data

Protein prep.†		N in prep.‡ (%)	Ash in prep.‡ (%)	Arginine N (%)		Histidine N (%)		Lysine N (%)
				Method 1	Method 2	Method 1	Method 2	
1E	<i>Phalaris tuberosa</i>	14.90	1.8	12.5 (5)	—	2.10 (4)	3.58 (2)	6.60 (3)
2E	<i>Hordeum murinum</i>	14.10	2.8	12.6 (4)	—	2.00 (4)	3.52 (3)	6.33 (4)
3E	<i>Medicago sativa</i>	14.15	2.3	12.2 (2)	12.4 (2)	2.16 (2)	3.65 (3)	6.36 (2)
4E	<i>Medicago denticulata</i>	14.55	1.4	11.6 (3)	—	2.00 (3)	3.86 (2)	6.51 (3)
'Within preparations' estimate of standard deviation				[0.42]	[0.14]	[0.229]	[0.034]	[0.303]

* In parentheses beside the values are indicated the numbers of estimations from which the values (the means) were derived.

† The serial cyphers are those assigned by Lugg & Weller (1944).

‡ Data from Lugg & Weller (1944).

Table 3. Percentages* of the protein N occurring as arginine, histidine and lysine, the values having been corrected for hydrolysis losses believed to result from the presence of impurity

Protein prep.		N in prep. (%)	Arginine N (%)	Histidine N (%)	Lysine N (%)
1E	<i>Phalaris tuberosa</i>	14.90	13.7 (5)	3.66 (2)	6.60 (3)
2E	<i>Hordeum murinum</i>	14.10	14.3 (4)	3.63 (3)	6.33 (4)
3E	<i>Medicago sativa</i>	14.15	14.0 (4)	3.76 (3)	6.35 (2)
4E	<i>Medicago denticulata</i>	14.55	13.0 (3)	3.97 (2)	6.51 (3)

Analysis of variance

Source of variation	Arginine			Histidine			Lysine		
	D.F.	S.S.	Mean square	D.F.	S.S.	Mean square	D.F.	S.S.	Mean square
Between preparations	3	3.21	1.07	3	0.1521	0.0507	3	0.1526	0.0509
Within preparations	12	2.24	0.187	6	0.0070	0.0012	8	0.7325	0.0916
Variance ratio			5.7			42			0.56

As judged by the *F* variance ratio, there is a significant difference between the mean squares for arginine, a highly significant difference between those for histidine, and no significant difference between those for lysine (see discussion).

* In parentheses beside the values are indicated the numbers of estimations from which the values (the means) were derived.

a uniform correction factor was applied to the weights of crude salt in most of the analyses. It is clear that method 2 has yielded consistently larger histidine values than method 1.

Even if the correction for overall losses (+2.1 mg. N) were not applied, the values for histidine obtained by method 2 would still be much greater than the corrected values by method 1. Obviously, the histidine values obtained by method 1 cannot be accepted. The only explanation which seems to be in keeping with the careful examination, which Tristram (1939) made, is that, whereas at the stage of precipitation of the histidine diflavinate his test solutions probably contained very little amino-acid other than histidine, those derived from the protein hydrolysates probably contained rather more, and that the solubility of the histidine salt must be vastly increased in the presence of the other amino-acids. Our test mixtures contained large quantities of other amino-acids, but in any case Vickery & Winternitz (1944) have adduced reasons for believing that the solubility of histidine-DBS is probably only slightly affected by the presence of other amino-acids. Attention must be drawn to the unusually small variability of replicate histidine estimations made by method 2.

There is some uncertainty about the accuracy of the lysine values. Chibnall, Rees & Williams (1943) obtained good agreement between lysine values for β -lactoglobulin estimated by Tristram's (1939) method and by their own

comprehensive method in which, likewise, lysine monopicate is weighed. However, a comparison of these values with those obtained by isotope dilution, microbiological assay, specific decarboxylase, and electro-dialysis (with N difference) methods, shows that the former are almost certainly too low (see Macpherson, 1946). Chibnall (1946) has given comparisons for some other proteins, the agreement being excellent in some instances and poor in others. In Tristram's (1939) method the initial precipitation of the lysine monopicate occurs under such conditions (high concentration of ethanol) that the solubility of the salt is probably very low, whether other picrates are present or not. Our assessment of the purity of the crude lysine monopicate was not based upon recrystallization losses. Consequently, if enhanced recrystallization losses constitute the major source of systematic error in such estimations, our values may be fairly reliable.

In compiling Table 3 we have returned to the individual values from which the mean values shown in Table 2 were derived. Of the histidine values only those obtained by method 2 have been used. All the arginine values have been used. Those for 3E obtained by both methods have been grouped, as they differed but little among themselves, and the inclusion of the two values obtained by method 2 could not appreciably affect a general statistical analysis based upon the supposition that all values were obtained in the same way. The individual values have then been corrected

on the following basis. The assumption has been made that during hydrolysis the protein preparations yielded all of these three amino-acids contained in them, that the destruction in the absence of carbohydrate or polyuronide impurities would not have been any greater than in the test 'hydrolysis' experiments (i.e. 0%), because the bulk of the amino-acids is probably liberated within the first several hours of hydrolysis (see Lugg, 1946), that the losses due to the presence of such impurity are proportional to the amount of it, that the amount can be calculated with sufficient precision from the known N contents of the preparations (corrected for ash) and the N contents (believed to be about 16.75%; see Lugg, 1939) of the hypothetical pure proteins, and that 1.00 g. of arabinose corresponds in quantity and effects with 0.88 g. of (pentosan) impurity (Lugg, 1939).

The impurities present in 2.5 g. quantities of the four preparations were thus calculated to have been equivalent in effects to the following quantities of arabinose: 0.27 g. (1E); 0.38 g. (2E); 0.39 g. (3E); 0.34 g. (4E). The corresponding corrections for the arginine and histidine values were calculated from the data in Table 1. On the basis of Tristram's (1939) work, no corrections were required for the lysine values.

Lugg (1939) was of the opinion that the common impurity in preparations like these may be pectin or more probably mucilage, rather than pentosan, and the corrections calculated on the above basis may require revision if, at some future time, recovery hydrolysis experiments are made with these other possible contaminants. The means of the corrected values are shown in Table 3. The corrected (individual) values were used in the analysis of variance given at the foot of the table.

DISCUSSION

Although an element of uncertainty must yet remain with respect to destruction of amino-acids during their hydrolytic liberation from proteins, recent extensive analyses of several 'pure' proteins (reviewed by Chibnall, 1946) suggest that the destruction, in most instances, is probably small. The results given in Table 3, in which corrections have been made for assessable losses, are to be viewed in the light of these remarks.

The arginine and lysine contents of the four protein preparations are of similar magnitudes to those found by Tristram (1939) for plant-leaf protein preparations which (for the most part) were probably less fully representative of the total protein in the leaves, but there is reason to believe that Tristram's (1939) arginine values may have been somewhat overestimated (see earlier remarks concerning methods and overall losses). The histidine contents of the four preparations are very considerably greater than those found by Tristram (1939) for the other preparations. It is to be noted, however, that when the method used by Tristram (1939) was applied to the four preparations, values similar to those he reported for the other preparations were obtained. For reasons already discussed, we have discarded the histidine values obtained by Tristram's (1939)

method. We are of the opinion that the histidine values reported by Tristram (1939, 1940), and by Lugg (1943) who used the same method in an analysis of protein from the fronds of *Pteridium aquilinum*, were probably too low. Indeed, it seems likely, in the light of the discussion in the previous section, that almost all protein histidine contents obtained by the use of methods involving precipitation as di-flavinate, whether they be large- or small-scale procedures, will require re-examination. This is implied also by the results of recent electrodialysis studies (Macpherson, 1946).

Attention has been drawn (Lugg & Weller, 1944) to the fact that the cyst(e)ine and methionine contents of the legume leaf proteins (3E, 4E) are appreciably lower than those of the grass leaf proteins (1E, 2E), and that the same tendency had been observed among protein preparations, many of which were probably far less fully representative of the total protein in the tissues. An interesting point arising from the present study is the astonishing uniformity in the arginine, histidine and lysine contents of the four preparations. Statistical examination of the values, with the aid of the analysis of variance given at the bottom of Table 3 and the *t* test, reveals that at the 95% probability level the differences in arginine values are significant only as between 4E and the other preparations, whereas the differences in histidine values, small though they are, are significant in all cases except as between 1E and 2E. However, the differences and their degrees of significance would be modified somewhat by the incidence of errors in the reported N contents of the protein preparations.

SUMMARY

1. The arginine, histidine and lysine contents of protein preparations, 1E, 2E, 3E and 4E, which are believed to be reasonably representative of the total protein in leaves respectively of *Phalaris tuberosa* L., *Hordeum murinum* L., *Medicago sativa* L., and *M. denticulata* Willd., have been estimated. As percentages of the protein N occurring in the forms of these three amino-acids, the values for the four plant species, respectively, may be expressed: arginine, 13.7, 14.3, 14.0, 13.0; histidine, 3.66, 3.63, 3.76, 3.97; lysine, 6.60, 6.33, 6.36, 6.51. The work involved an examination of methods of analyzing hydrolysates, and an examination of the probable effects of impurities in the protein preparations upon the analyses.

2. It is believed, on the evidence afforded by fractional recrystallization of amino-acid picrates and 'one-dimensional' partition-chromatographic analyses of the fractions, that the preparations contained no hydroxylysine.

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Protein in Senescent Leaves of *Trifolium subterraneum*: Partial Amino-acid Composition

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It has been shown (Lugg & Weller, 1941) that in very young leaf tissue the whole protein composition may differ rather markedly from that of the whole protein in older tissue. (The axial organs of germinating seeds were used, but the bulk of the protein was present in the stalks and plumules.) In the following account it will be shown that at the other extreme of age (senescent tissue) the departure in composition of the whole-leaf protein from the pattern which seems to prevail during most of the life of the plant may again be fairly marked in some respects.

EXPERIMENTAL

Preparation of materials. The fresh plant material consisted of the leaves and attached petioles of *Trifolium subterraneum* harvested very late in the season (December, 1940) from senescent plants growing in a relatively cool and damp environment in the Adelaide foothills. 'Yellowing' was somewhat advanced in about a third of the leaf material, and much of the rest showed traces of it.

From the bulk of the fresh material an 'extracted' protein preparation (E) was made by the alkaline buffer lipid-

solvent method (Lugg, 1939) in the manner described by Lugg & Weller (1944). 'Residual' (R) and 'whole' (W) protein preparations were also made by methods previously described (Lugg, 1939).

Data concerning the fresh material and the protein preparations made from it are shown in Table 1. The terms 'coagulable N' and 'protein N' have been explained fully in earlier publications (e.g. Lugg, 1939). For the rest, the table is self-explanatory.

Analytical work. Nitrogen estimations were made by the Kjeldahl method with Se catalyst. The amide, tyrosine, tryptophan, cyst(e)ine (i.e. cysteine plus cystine) and methionine contents of the protein preparations, W, E and R, were estimated by methods previously described (Lugg, 1937; 1938a, b, c; Lugg & Weller, 1941). The 'differential oxidation method' was used for the estimation of the cyst(e)ine and methionine contents. Considerable difficulty was encountered with the tryptophan estimations on the W and R preparations because of the unusually intense brown coloration of the alkali-stannite hydrolysates. Corrections for this coloration were so large that the precision of the estimations was seriously impaired.

The arginine, histidine and lysine contents of preparation E were estimated by methods recently described (Lugg & Weller, 1948) and based upon the procedures of Tristram (1939) and of Vickery & Winternitz (1944). These methods are considered to be unsatisfactory for use with preparations

Table 1. *Extraction of protein from senescent leaves of Trifolium subterraneum*

Fresh wt. taken (kg.)	Dry wt. (%)	N in dry leaf (%)			Extracted N (%)			Protein pre- paration	N in prep. (%)	Ash in prep. (%)
		Total	Coagulable	Protein	Total	Coagulable	Protein			
12.3	20	2.45	2.05	1.99	—	—	—	W	3.16	2.4
—	—	—	—	—	33	28	26	E	13.15	2.6
—	—	—	—	—	—	—	—	R	2.10	3.5

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