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The Amino Acids of Cytoplasmic and Chloroplasmic Proteins of Barley

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During the germination and the early phases of development of barley plants there is a rapid synthesis of protoplasmic proteins, particularly in the young leaves and root system of the seedlings (Folkes, Willis & Yemm, 1952). These proteins form an integral part of the protoplasts of the cells, and ultimately most of the nitrogen assimilated by the plant is incorporated in cytoplasmic and chloroplasmic structures. The data reported in this paper were collected in the course of an investigation into the relation between the synthesis of the protoplasmic proteins and the metabolism of amino acids in the young seedling.

Extensive data have been published on the protoplasmic proteins of various plants and have been reviewed by Chibnall (1939) and Lugg (1949). Yemm (1950) gave an account of the separation and partial analysis of preparations containing a mixture of chloroplasmic and cytoplasmic proteins from mature leaves of barley. This work has now been extended to a similar preparation from barley seedlings and to a preparation from mature leaves which consists mainly of cytoplasmic components. Moreover, in the present analyses a more comprehensive investigation of the constituent amino acids in these preparations has been made; analytical data, obtained chiefly by microbiological assay, are recorded for eighteen amino acids.

A short account of the work has already been given to the Biochemical Society (Yemm, 1951; Folkes & Yemm, 1951).

METHODS

Preparation of proteins

Mixed protoplasmic proteins of seedlings. Seedlings of barley were grown by soaking in water overnight 500 g. of grain (N, 2.5% of dry weight) and sowing in moist sand in a cold frame at about 15°. After 14 days the seedlings were uprooted, washed free from sand, and squeezed fairly dry. All the seedlings (total fresh weight, 4.5 kg.) were used for the preparation of protein by a modification of the 'used ether-water' method of Chibnall, Miller, Hall & Westall (1933). Batches (500 g.) of seedlings were treated serially, cytolysed for 15 min. in 1 l. ether water and then pressed in a screw-plunger press, the expressed juices being returned to the ether water which was used for the next batch. The residue from the press was washed in 500 ml. tap water, pressed again, minced finely in a domestic mincer and extracted twice with 0.025M-Na₂B₄O₇ at pH 9.2 (Lugg, 1939), 500 ml. each time, the suspension being passed through a 1 mm. mesh wire sieve. The suspension was centrifuged at 400 g for 5 min. and then filtered through a pad of rammed paper pulp, 3 cm. thick. The combined filtrates were adjusted to pH 4.4 by the addition of about 190 ml. 2N-HCl. The protein was coagulated by heating the solution on a water bath to 80° for 5 min. and allowed to settle overnight in a large vessel. Most of the clear supernatant was then decanted and the protein was separated by centrifuging at 1500 g for 10 min. The preparation was extracted three times at the centrifuge with 0.005N-HCl, as recommended by Lugg (1938), and twice with 300 ml. absolute ethanol, held at 60° on a water bath for 30 min. After two washes with dry ether the preparation was dried at 80° in a ventilated oven and ground to a powder (5.09 g.).

Mixed protoplasmic proteins of mature leaf. This preparation has already been described by Yemm (1950) as specimen E1. The protein was obtained from mature leaves of barley grown under field-crop conditions, by a method similar to but not identical with that described above.

Chloroplastic proteins of mature leaf. The upper three or four leaves of barley plants not yet in ear were used for this preparation. The material (2.5 kg.) was cut into short lengths, washed with water, shaken free from adhering moisture and minced into 3 l. distilled water. The slurry was passed through a 2 mm. mesh sieve and the fibrous solid washed twice with 1 l. distilled water. The extract and washings (5 l.) were centrifuged at 150 g for 2 min. and the residue washed twice at the centrifuge with 200 ml. water. This residue was found on microscopic examination to consist mainly of cell-wall debris with a few chloroplasts and was rejected. The centrifuged extract and washings were stored overnight at 4°. During this time a slight flocculation of the chloroplast granules occurred and was completed in the morning by the addition of 300 ml. 2M-CaCl₂ as recommended by Neish (1939). The flocculated chloroplasts were collected at the centrifuge (750 g for 15 min.), washed twice and then sucked dry on a filter paper on a Büchner funnel and dried at 80°. Microscopic examination showed this chloroplastic fraction to be free of cell-wall material. Its weight was 27.4 g.

Cytoplasmic proteins of mature leaf. The solution from the centrifuge in the above preparation was filtered by suction through a well-rammed pad of paper pulp, 2 cm. thick, to remove all traces of chloroplasts. The protein was precipitated at pH 4.4, allowed to settle overnight, collected by decanting and centrifuging, washed with absolute ethanol and ether and dried. This cytoplasmic fraction weighed 4.16 g.

Gladiin and edestin. These were specimens of preparations already subjected to analysis by Dr A. C. Chibnall and his collaborators.

Analysis of the protein preparations

As suggested by Chibnall, Rees & Williams (1943), the preparations were allowed to come to moisture equilibration with the air before being used for analysis.

Total nitrogen. A semimicro modification of the Kjeldahl method of Chibnall *et al.* (1943) was used. The preparation (10–20 mg.) was heated for at least 8 hr. with 0.5 g. catalyst (K₂SO₄, 80 g.; CuSO₄, 5H₂O, 20 g.; SeO₂, 0.3 g.), 2 ml. H₂SO₄ and 1 ml. water. The solution was treated with 12 ml. 7.5N-NaOH and steam-distilled in the conventional manner into 5 ml. 0.05N-H₂SO₄. The distillate was made to 100 ml. and 10 or 20 ml. samples were taken for the estimation of ammonia using Nessler reagent. Since this method is insensitive to low concentrations of ammonia (Wirth & Robinson, 1933), 5 ml. 0.00015M-(NH₄)₂SO₄ was first added to each sample, followed by 3 ml. Nessler reagent (Vanselow, 1940) and distilled water to make to 50 ml. After 15 min. colour intensity was measured in a Spekker absorptiometer using 2 cm. cells and a 430 mμ. filter (Ilford no. 601) with distilled water as the reference standard. A complete reagent blank was included in each set of estimations, and results were calculated from a standardization curve which was determined daily using a standard (NH₄)₂SO₄ solution.

Amide nitrogen. The method of Yemm (1950) was used with the following modifications: 18–20 mg. of the preparation was refluxed for 3 hr. with 5 ml. 2N-H₂SO₄ and the

ammonia liberated was vacuum distilled into 1.5 ml. 0.05N-H₂SO₄. The whole distillate was used for colorimetric estimation of ammonia as described above.

Ash. (Determined by Mr W. W. Eno.) Portions (3–12 mg.) were incinerated at 900° to constant weight.

Ether- and ethanol-soluble lipids. The preparation (0.5–1.3 g.), dried at 80°, was extracted in a Soxhlet apparatus to constant weight, first with ether and then with an ethanol: ether (3:1, v/v) mixture. The loss in weight was ascribed to lipid. The N content of the extract was estimated from total-N determinations on the extracted residues.

Moisture. Samples (20–50 mg.) in glass microweighing bottles were dried to constant weight in an electric oven at 105°.

Estimation of amino acids

Most of the analyses were made on acid hydrolysates of the proteins, but tryptophan was estimated in alkaline hydrolysates and both acid and alkaline hydrolysates were used for the estimation of tyrosine because of uncertainty as to its stability (see Block & Bolling, 1945, p. 81).

Hydrolysis. The method of Henderson & Snell (1948), of autoclaving 100–500 mg. protein in a sealed tube with 3N-HCl or 5N-NaOH, was adopted. The sealed tubes were immersed in a water bath for about 30 min. with periodic shaking and then autoclaved at 15 lb./sq.in. (120°) for 6–7 hr. with acid or 15 hr. with alkali. After cooling, each solution was adjusted at the glass electrode to pH 6.8 with either 5N-NaOH or 3N-HCl. Some of the acid hydrolysates were then filtered through an asbestos pad and the insoluble humin, after washing three times with a little warm distilled water, was taken for Kjeldahl estimation of the humin N. The filtrate and washings were made up to 25–100 ml.; the alkaline hydrolysates tended to form a gel at this concentration and were diluted to 250 ml.

Ammonia N. The ammonia released on acid hydrolysis was determined by distillation, as described above, of 3–4 ml. portions of the hydrolysates.

Microbiological assay. The assays were carried out by the method of Henderson & Snell (1948) with some modifications. Flat-bottomed glass tubes, 10 cm. × 2.5 cm., were used with a total volume of 2 ml. of solution in each tube. The protein hydrolysates (suitably diluted) or amino acid standards were pipetted out in volumes from 0.2 to 1.0 ml. and water added when necessary to make 1 ml. The basal medium (1 ml.), deficient in the amino acid being assayed, was run into each tube. Five replicates of each level of standard (1.0, 0.8, 0.6, 0.4, 0.2 ml.) and of the blanks (1 ml. water + 1 ml. basal medium) were generally set up, together with three replicates at each of the five levels of protein hydrolysate. The tubes, in groups of 12, were supported in copper racks, each fitted with a deep aluminium lid padded with cotton wool. In this way individual plugging and labelling were avoided. The replicates were distributed throughout the racks so as to minimize systematic errors due to differences in conditions during sterilization and incubation.

The tubes were sterilized at 10–12 lb./sq.in. for 10 min., inoculated with a suspension of the assay organism, and incubated at 35° for 60–72 hr. in a humid atmosphere. The lactic acid present at the end of this time was estimated by electrometric titration against 0.04N-NaOH to pH 7.3. A compact assembly of a 15 ml. automatic burette, glass electrode (Cambridge Instrument Co.) and a saturated KCl-agar bridge dipped into the culture solution which was

stirred during titration by means of a stream of CO₂-free air. Distilled water (2 ml.) was added to each tube and the NaOH was then run in rapidly until pH 6.8 was reached, after which cautious additions brought the pH to 7.3.

The mean titration for each level of the standard and for the blank were plotted against amino acid concentration to give a standard curve. From this curve the concentration of amino acid equivalent to the mean titration for each level of protein hydrolysate was read off and five values of the amino acid content of the protein could thus be calculated.

The assay organisms were *Lactobacillus arabinosus* 17-5, *Leuconostoc mesenteroides* P-60 and *Streptococcus faecalis* R. They were obtained from the Biochemistry Department of the College of Agriculture, University of Wisconsin. Immediately on arrival, the cultures were used to inoculate 100 ml. batches of the enriched basal medium of Henderson & Snell (1948) and the resulting cultures were centrifuged aseptically, resuspended in the sterile dextrin-ascorbic acid medium of Naylor & Smith (1946), and freeze-dried in small ampules. A small portion of the dried culture was used to inoculate 5 or 10 ml. volumes of enriched basal medium, the culture resulting from 8 to 24 hr. incubation being centrifuged, washed, and suspended in sterile 0.9% (w/v) aqueous NaCl for use as an inoculum.

The amino acids used as standards were given by Glaxo Laboratories Ltd. They were dried *in vacuo* at 40° before use. Total nitrogen estimations by the Kjeldahl method gave values agreeing with the theoretical N content within the experimental error of the determinations. The natural (L) forms of amino acids were used with the exception of aspartic acid, proline, valine, isoleucine, phenylalanine, tryptophan, threonine and methionine, where only DL-forms were available. In most of these only the L-form was utilized by the organism so that double the concentration of DL standard was required. Both forms of aspartic acid were utilized. DL-Tryptophan was used as standard to allow for the complete racemization of this amino acid during protein hydrolysis with NaOH; tyrosine is similarly racemized under these conditions and corrections were made accordingly in the calculations.

The basal medium of Henderson & Snell (1948) was used except for the assay of glutamic acid, when aspartic acid was replaced by half the quantity of asparagine, as recommended by Brickson, Henderson, Solhjell & Elvehjem (1948). This avoided lag or induction effects at low concentrations of glutamic acid. Similar effects in the assay of proline, valine and leucine were avoided by the use of larger inocula and by adjusting the medium to pH 6.0. This effect emphasized the importance of pH and led to the practice of carefully adjusting the basal medium, standard, and protein hydrolysates to the same pH value before setting up an assay. Commercial supplies of amino acids were found to be sufficiently pure for use in the basal medium except in the assay of glycine. Henderson & Snell (1948) state that recrystallization of alanine was necessary, but this did not sufficiently reduce the blank to make an assay possible. Heathcote (1948) recommended recrystallization of the threonine used, and this was found effective, although the blank in this assay was still higher than in any other.

Henderson & Snell (1948) did not attempt the assay of cystine by their procedure. Shankman (1943) showed that *Lb. arabinosus* was sensitive to cystine nutrition, but attempts in this laboratory to use this organism were unsuccessful. Heathcote (1948) reported that he used *Ln. mesenteroides* successfully for cystine assay, although some

variability in the standard curves was experienced. We have found that this can be largely avoided if the assay is carried out at pH 6.0, and the results given here are by this method. *Strep. faecalis* has proved far less dependable in our hands than the other two organisms, and in some cases no growth has been obtained in the assay tubes. Attempts have therefore been made to assay arginine, threonine and methionine, usually determined with this organism, by means of *Ln. mesenteroides*. These were successful with methionine and partially so with arginine, although in this assay it was noted that the values obtained were about 10% higher than those given by *Strep. faecalis*, and that a systematic increase or decrease with increasing protein concentration sometimes occurred. Threonine gave erratic assays and was eventually determined chemically. The assay organisms were therefore used for the estimation of amino acids as follows: *Lb. arabinosus* for glutamic acid, leucine, valine, phenylalanine and tryptophan; *Ln. mesenteroides* for aspartic acid, proline, glycine, isoleucine, tyrosine, serine, cystine, methionine, lysine and histidine; *Strep. faecalis* for arginine.

Estimations with glutamic acid decarboxylase. In order to test the estimates of glutamic acid made by microbiological assay, determinations were also made by the specific decarboxylase method of Gale (1945). The decarboxylase used was a suspension of washed cells of *Clostridium welchii* S.R. 12 prepared and tested in this laboratory by Willis (1950) who showed it to be specific for glutamic acid; it yielded no CO₂ with seventeen other amino acids tested. This preparation (0.5 ml.; 12.5 mg. dry weight of bacteria) was used for each determination, in the side bulb of the Warburg flask. Protein hydrolysate (0.6–2.0 ml.; depending on glutamic acid content), together with 1 ml. 0.6M sodium acetate buffer (pH 4.9) and distilled water to make to 3 ml., were pipetted into the flask. CO₂ evolution after tipping in the decarboxylase preparation was measured in Warburg manometers at 35°, corrections being made for CO₂ evolved from a control flask containing no hydrolysate.

In order to obtain rapid and complete evolution of CO₂ from some of the protein hydrolysates it was necessary to add Cetavlon (cetyltrimethylammonium bromide, Imperial Chemical (Pharmaceuticals) Ltd., Manchester) in the 0.6M-acetate at 0.15% (w/v) as recommended by Hughes (1949). Tests with pure glutamic acid showed that only 96% of the theoretical yield of CO₂ was obtained. This was assumed to be owing to traces of D-glutamic acid formed during preparation of the amino acid. As a similar degree of racemization may be expected in the protein hydrolysates (Chibnall, Rees, Williams & Boyland, 1940), a correction based on 96% yield was applied.

Estimation of alanine and threonine. Alanine and threonine were determined by colorimetric estimation of the acetaldehyde produced on oxidation of the hydrolysates with ninhydrin and periodic acid respectively (Folkes, 1953).

RESULTS

General analyses

General analyses have been performed on the cytoplasmic and chloroplastic preparations from mature leaves and on the preparations of mixed protoplasmic proteins both from mature leaves and from seedlings. The results are given in Table 1. The values for total nitrogen are the mean of two or more estimates which differed by less than 1%, and are corrected for moisture which was determined on duplicate samples. Ash contents are, in general, based on a single

Table 1. *Analysis of protein preparations*

	Protoplasmic proteins					
	Gliadin	Edestin	Seedling mixed	Mature leaf		
				Mixed	Cytoplasmic	Chloroplastic
	(% Moisture-free preparation)					
Total N	17.6	18.8	14.0	14.0	14.6	9.0
Total N (Chibnall, 1942)	17.66	18.65	—	—	—	—
Ash	—	—	1.2	1.2	1.5	6.6
Ether- and ethanol-soluble lipids	—	—	—	—	0.4	21.4
Protein (insol. N × 5.9)	—	—	—	—	86.1	51.5
Other organic matter	—	—	—	—	12.0	20.5
(by difference from 100 %)						
	(% Ash- and moisture-free preparation)					
Total N	—	—	14.2	14.2	14.8	9.6

determination but duplicates agreed closely. Estimates of lipid material were made only for the cytoplasmic and chloroplastic preparations from mature leaves; in these cases it is possible to calculate the probable content of other non-protein material by assuming all the insoluble nitrogen to be present as protein. The use of the factor ($\times 5.9$) for this calculation is discussed later.

Amino acid content

Estimations of constituent amino acids have been obtained on all the preparations with the exception of the chloroplastic preparation which, as shown in Table 1, is too contaminated with non-protein material for accurate analysis. The results for the other preparations are given in Table 2.

It is possible to assess the accuracy of the amino acid estimates in a number of ways from the data obtained in the analyses. In the microbiological assay the reliability is indicated by the degree of agreement between the estimates based on the five levels of protein hydrolysate, each the mean of three tests. In a satisfactory assay these estimates agree closely and do not change systematically with increasing protein level. All the assay results quoted in Table 2 with the exception of those of arginine content, are derived from assays in which the maximum deviation from the mean of the five estimates is less than 10 % of the mean value, and in many of the assays the maximum deviation is between 3 and 4 %. The precision of the mean value cannot be calculated with certainty however from these deviations owing to non-normal distribution of errors which sometimes occurs. It has been found that slight differences in pH of the standard solution and of the hydrolysate may give rise to errors of this type. Under such conditions induction effects may cause a bad fit between the unknown and the standard curve. In severe cases these effects are obvious from the systematic change in titre with increasing level of hydrolysate, and the results can be discarded, but in some cases they are small and not immediately apparent. Another source of error may be inhibitory or antagonistic effects shown by high concentrations of the hydrolysate. For instance, in the assay of lysine in gliadin it was found that the estimates of lysine N as % total N of the hydrolysate, based on 0.2, 0.4, 0.6, 0.8 and 1.0 ml. levels of hydrolysate, were 0.66, 0.62, 0.58, 0.49 and 0.45 respectively. This hydrolysate was used undiluted because of the low level of lysine present, and the high concentrations of other substances had obviously interfered with the assay. The best estimate of lysine content here was obtained by extrapolating a graph through the points to zero level of hydrolysate, giving a value

of 0.68–0.74 %. With the limitations outlined above, the assays are satisfactory and the standard error of all determinations (except arginine) is less than ± 4 %.

A better estimate of the accuracy of the assays can be obtained by comparison of the results of duplicate analyses. Relatively few assays were repeated unless the first one was unsatisfactory, but in all cases where both assays were satisfactory (indicated by an asterisk in Table 2) the difference between the two results was less than 3 % of the mean value. In a few instances the reliability of estimates of amino acid content may be judged by comparing the results of different methods of analysis. The determinations of glutamic acid by assay and decarboxylase methods are valuable in this respect and the results obtained on several proteins are included in Table 2. It will be seen that agreement is satisfactory; the largest difference is 5 % of the mean and for gliadin and for edestin the differences do not exceed 1 %.

The chemical analyses of gliadin and edestin by Chibnall and his colleagues (collected by Tristram, 1949) offer a further test of the validity of the assays and the results are shown in Table 2. In these proteins only the dicarboxylic and basic amino acids were estimated by microbiological assay. The results for glutamic acid are in close agreement, but for the other amino acids the assay gives generally somewhat higher values than are obtained by direct chemical isolation. The largest differences are in the estimates of aspartic acid and here the results of other workers using microbiological assay (Tristram, 1949) indicate that this amino acid in particular was underestimated by isolation methods. The assay of arginine in gliadin and edestin was made with *Ln. mesenteroides* and as this organism has been found to give values approximately 10 % higher than are given by *Strep. faecalis* it is probable that in this case the discrepancy may be due to assay errors. The arginine contents of the protoplasmic proteins were determined with *Strep. faecalis* and are considered more reliable although not entirely satisfactory.

Replicate analyses of threonine on different occasions and at different levels of hydrolysate showed quite close agreement, the largest variation being 5 % of the mean with the tissue proteins and 13 % with gliadin and edestin. The mean values obtained for these latter proteins agree well with those found by Rees (1946) using a macromethod (see Table 2). The replicate analyses for alanine showed small variability, but their reliability may be less because of the necessity for a correction, amounting to 15 % of the total, for the interference of other amino acids.

Table 2. *Amino acid content of the protein preparations*
(All amino acid results as amino acid N in % of protein N.)

	Gliadin			Edestin			Protoplasmic proteins				
	Others (Tristram, 1949)		Present work	Others (Tristram, 1949)		Seedling mixed	Leaf			Gramineae (Lugg, 1949)	<i>Chlorella</i> (Fowden, 1952)
	Present work						Mixed	Cytoplasmic			
Amide (mild hydrolysis)	25.0	25.3	9.5	9.50	4.85	4.75	4.80	6.1	6.4	4.7-5.3	6.1
Aspartic acid	1.4	0.86	8.0	6.75	6.6*	6.6	6.4	6.4	7.8	4.9-5.4	6.4
Glutamic acid {assay	25.6}	25.3	{10.3}	10.55	{8.0}	8.2	8.2	7.2	7.2	6.6-7.8	7.8
{decarboxylase	25.3}		{10.2}		{7.9}	7.8	7.9	4.1*	4.1*	3.1	7.2
Proline	—	—	—	—	3.9*	4.0*	6.7	6.2	6.2	0.4	6.2
Glycine	—	—	—	—	6.9	6.5	7.9	7.7	7.7	4.4-5.1	7.7
Alanine	—	—	—	—	8.3	8.0	4.9	5.5	5.5	3.3-4.2	5.5
Valine	—	—	—	—	5.1	5.0	5.9	{6.1	{6.1	7.1-8.8	{6.1
Leucine	—	—	—	—	6.0	5.8	4.2	{3.5	{3.5	2.5-2.6	{3.5
Isoleucine	—	—	—	—	4.5	4.7	3.0	2.8	2.8	2.3-2.5	2.8
Phenylalanine	—	—	—	—	3.1	3.2	2.0	2.1	2.1	1.8-2.1	2.1
Tyrosine {acid hydrol.}	—	—	—	—	{2.3}	2.2	3.5	3.3	3.3	—	3.3
{alk. hydrol.}	—	—	—	—	{2.4}	2.2	4.0	4.0	4.0	3.0	2.9
Tryptophan (alk. hydrol.)	—	—	—	—	1.9	1.8	0.9	0.9	0.9	1.3-1.5	0.2
Serine	—	—	—	—	3.5	3.3	1.4	1.4	1.4	1.4-1.6	1.4
Threonine	1.4	1.40	2.4	2.43	4.0	4.0	7.1*	7.1*	7.1*	6.3-6.6	10.2
Cystine + cysteine	—	—	—	—	0.9	0.9	4.1*	4.1*	4.1*	3.6-3.7	3.3
Methionine	—	—	—	—	15	15	15	15	15	13.7-14.3	15.8
Lysine	0.7	0.70	2.8	2.5	4.3	2.4	3.0	3.0	3.0	—	0.0
Histidine	3.2	2.80	4.2	4.2	5.8	6.0	5.8	5.8	5.8	—	—
Arginine	5.4	5.00	31.0	28.8	101.5†	97.4†	98.2†	98.2†	98.2†	74.4 (mean)	101.3
Insoluble humin	—	—	—	—	—	—	—	—	—	—	—
Ammonia (acid hydrol.)	—	—	—	—	—	—	—	—	—	—	—
Total in acid hydrolysate	—	—	—	—	—	—	—	—	—	—	—

* Based on duplicate assays.

† Amide and tryptophan are omitted from this total. A mean value has been taken for glutamic acid.

The estimates of analytical errors given above take no account of losses of amino acids during hydrolysis of the protein preparations. In the case of the tissue proteins which are relatively impure and contaminated with material of a carbohydrate nature these losses may be severe in some cases (see Lugg: appendix to Chibnall, 1939). No direct test of the degree of destruction with varying hydrolysis procedures has been made in the present study except in the case of tyrosine. Here both acid and alkaline hydrolysates were assayed and the results are given in Table 2. The two series of results agree quite closely but the acid hydrolysates gave generally somewhat lower values; this probably indicates slight destruction during acid hydrolysis, but does not eliminate the possibility of partial destruction under both acid and alkaline conditions. That destruction of some amino acids occurs under acid hydrolysis of the protoplasmic proteins is shown by the relatively large amounts of insoluble humin formed during the process. While this humin is largely derived from carbohydrates or other contaminants, the nitrogen associated with it originates from amino acids (Chibnall, 1939). Tryptophan is of course totally destroyed on acid hydrolysis and partial destruction of cystine and cysteine, and to a lesser extent methionine, also occurs, especially when hydrolysis is carried out in the presence of carbohydrate contaminants (Bailey, 1937; Lugg: appendix to Chibnall, 1939). All four of these amino acids may therefore contribute to the humin N. A further index of amino acid destruction is the production of ammonia during vigorous acid hydrolysis in excess of that liberated from the amide groups by mild hydrolysis. This excess ammonia is probably formed by partial destruction of the hydroxyamino acids, serine and threonine (Rees, 1946). The degree of destruction indicated by these figures is, however, small in relation to the total amino acid content, and it is encouraging in this connexion to note that the compounds determined in the acid hydrolysates of the protoplasmic proteins account for between 98 and 100% of the total nitrogen. This is within the experimental error of the determinations and suggests that extensive destruction of amino acids has not occurred under the conditions of hydrolysis used here.

The comparatively complete recoveries of total nitrogen of the protoplasmic preparations make it possible to calculate the nitrogen content of the protein assumed free from carbohydrate or other matter. The amino acid and amide residues account for 96–98% of the total nitrogen and 80–84% of the moisture- and ash-free matter in the three preparations. The 'pure' proteins can be calculated to have a nitrogen content of 16.9–17.0% of their dry weights. This value agrees fairly well with that of 16.7–16.8% calculated by Lugg (appendix to Chibnall, 1939) for cocksfoot-grass protein and based on humin formation. The amount of contaminant in impure preparations can thus be calculated by assuming that protein equals $N \times 5.9$ (equivalent to N content 16.95%). This has been done in Table 1.

DISCUSSION

In addition to the proteins associated with the cytoplasm and the chloroplasts of green leaves, much smaller amounts of protein are found in the nuclei and possibly in the vacuole. Only the first two components are likely to be represented in preparations by the 'used ether-water' method (Chibnall, 1939).

In this method the chlorophyll-protein complex of the chloroplasts is disrupted and the chloroplastic proteins go into solution with the cytoplasmic proteins, a process which is even more efficient in the presence of alkaline buffers (Lugg, 1939). In the absence of ether-water the chloroplasts are not dispersed to any considerable extent and it is possible to separate them from the cytoplasm by centrifuging or precipitation. The analyses given in Table 1 show that this separation was largely achieved in the present investigation, and that big differences in the content of lipids and other organic matter (carbohydrates?) exist between the chloroplastic and cytoplasmic preparations. It is unlikely that the preparations are entirely free of the other component; the lipid/protein ratio of the chloroplastic preparation is lower than that found by Chibnall (1939) for spinach chloroplasts and the cytoplasmic preparation does contain a little lipid material. Nevertheless, it is clear that the two preparations are derived in the main from different cellular structures, and it is unfortunate that the degree of contamination of the chloroplastic fraction by non-protein matter was so high as to make reliable analyses, and hence a direct comparison of amino acid content of the two preparations, impossible. However, any major differences in the two components should be reflected by marked differences between the cytoplasmic preparation and mixed preparations by the used ether-water method.

Consideration of the results presented in Table 2 shows that differences between the protoplasmic preparations from barley are very small and almost all within the precision of the analyses, especially when allowance is made for the possibility of variable losses during the hydrolysis of the three preparations. Only the lysine estimates show marked differences which are statistically significant, being based on duplicate microbiological assays. Moreover, lysine is particularly resistant to destruction during protein hydrolysis (Chibnall, 1939) so that it is probable that this represents a real difference in composition of the proteins, although the reproducibility with different preparations and hydrolysates has not been investigated. It is of interest that lysine was the only amino acid found by Chibnall (1939; see also Lugg, 1949) to differ significantly in content between cytoplasmic and mixed protein preparations from spinach leaves.

The analyses of leaf proteins by other workers have been collected by Lugg (1949), and the range of values for each amino acid in various members of the Gramineae (including *Hordeum murinum*) are included in Table 2. These analyses, by chemical procedures, suffer from the defects already briefly discussed, particularly with regard to the aspartic acid values. The widest differences are found in the

monocarboxylic acids such as glycine and alanine where the chemical methods depending on esterification and distillation are generally conceded to be inaccurate. Lugg's values for cystine and cysteine are higher than those found in the present investigation and are likely to be more reliable as no special precautions were taken to avoid or correct for losses during hydrolysis in the present analysis.

It is of interest that these analyses of protoplasmic proteins of barley agree closely with those of the bulk protein of *Chlorella* recently reported by Fowden (1952) who used mainly chromatographic techniques (see Table 2). From a phylogenetic standpoint the correspondence is remarkable and it seems that there are few significant differences in amino acid content between the protoplasmic proteins of the Chlorophyceae and the Angiospermae (cf. Lugg, 1949). Amide, proline and lysine show the largest differences, while threonine and cystine may also present important variations.

It is difficult at present to assess the significance of these results in terms of the identity and nature of the protoplasmic proteins. There is little doubt that preparations such as those described here consist of mixtures of enzymic and structural proteins of the protoplasm, but little progress has yet been made in their separation and characterization by physical methods. Wildman & Bonner (1947) found that 75% of the cytoplasmic protein from spinach leaves gave a single electrophoretic peak, although Bonner (1950) reports that similar preparations from oat and pea seedlings were much more heterogeneous. On the other hand, a remarkable similarity of amino acid content seems to extend not only to proteins from different cellular structures but also to mixed preparations from widely different species. In barley there is little evidence of extensive changes in composition in preparations from young seedlings and from mature leaves or in cytoplasmic as distinct from chloroplastic proteins. As discussed by Lugg (1949), the bulk proteins of the cell, in contrast to other classes of plant proteins, form a group having relatively constant chemical composition as judged by amino acid content, but varying in physical and biological properties. However, the possibility must not be overlooked that the electrophoretically homogeneous protein fraction studied by Wildman & Bonner is a major component of both cytoplasmic and chloroplastic structures and overshadows the more variable composition of other constituent proteins.

A field in which these results are of more immediate value is that of plant metabolism. It is now possible to estimate more completely the amino acid requirements of growing plant tissues actively engaged in the synthesis of protoplasmic proteins. Moreover, the relative amounts of the various amino acids required for this synthesis would appear to be fairly constant, irrespective of the age of the plant or the proportion of cytoplasm to chloroplasts being laid down. Use of this fact and of the data presented here has been made in an investigation of the amino acid metabolism of germinating barley (Folkes, 1951), and a fuller account of these studies will be reported elsewhere.

SUMMARY

1. Methods are described for the preparation and analysis of representative samples of cytoplasmic and chloroplastic proteins of mature leaves and seedlings of barley. The amino acid analyses were mostly done by microbiological assay.

2. The reliability of the analytical methods employed is gauged by a consideration of the reproducibility of the results and by a comparison of the results of test analyses of gliadin and edestin with those reported by other workers. It is probable that in most cases the error of the analyses was less than $\pm 4\%$.

3. Estimates are given for the amounts of eighteen amino acids, together with amide N, ash, lipid and moisture, present in a preparation of cytoplasmic proteins separated from mature leaves, and in mixed preparations of cytoplasmic and chloroplastic proteins separated from either mature leaves or seedlings.

4. A comparison of these three preparations indicates a high degree of uniformity in their constituent amino acids; only in their lysine content do they show appreciable differences. The significance of these findings is discussed in relation to the results of others.

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Further Observations on Phrenosinic Acid

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In an earlier paper dealing with the fatty acids of phrenosin and kersin we advanced the view that phrenosinic (cerebronic) acid was a mixture of 85% α -hydroxy-*n*-tetracosanoic acid and 15% α -hydroxy-*n*-hexacosanoic acid with perhaps a small amount of α -hydroxy-*n*-docosanoic acid, thereby disposing, as we thought, of the rival claims of Levene, that it was α -hydroxyisopentacosanoic acid and of Klenk, that it was α -hydroxy-*n*-tetracosanoic acid (Chibnall, Piper & Williams, 1936). This conclusion was based on evidence derived from three sets of observations. (1) Comparison of the melting point and X-ray crystallographic data with those of a series of synthetic ($\pm$)- α -hydroxy-*n*-fatty acids from C_{13} to C_{18} (higher members were not available at the time) and of various mixtures thereof. (2) Comparison of the melting point of the acetyl derivative with those of the synthetic series mentioned above. (3) Comparison of the melting point and X-ray data for the *n*-fatty acid given on oxidation with those of synthetic *n*-fatty acids from C_{21} to C_{27} and of mixtures thereof.

We were aware, however, that our finding left

unexplained three observations to which attention was drawn at the time, but which were not emphasized because they had no immediate bearing on the point then at issue. First, phrenosinic acid, as normally prepared, always separates in the 'cauliflower-like' masses first described by Thudichum (1881), and shown later by Rosenheim (1916) to be conglomerates of irregular spherocrystals. Such a product gave an X-ray photograph that was difficult to interpret. We were at a loss to explain this phenomenon, for according to our experience, a similar mixture of synthetic α -hydroxy-*n*-fatty acids would crystallize in rhombic plates with rather broken edges. Secondly, the molecular weight, as determined by titration, was too high for the postulated mixture, as also were the analytical figures for carbon and hydrogen, suggesting the presence of some, probably non-homologous, impurity which was destroyed on oxidation. Thirdly, the melting point of the acetyl derivative, 66.5° , seemed low, even for a mixture, because the extrapolated value for ($\pm$)- α -acetoxy-*n*-tetracosanoic acid was about 83° .