XIII. THE REPRESENTATIVENESS OF EXTRACTED SAMPLES AND THE EFFICIENCY OF EXTRACTION OF PROTEIN FROM THE FRESH LEAVES OF PLANTS; AND SOME PARTIAL ANALYSES OF THE WHOLE PROTEINS OF LEAVES

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ALTHOUGH from the time of Rouelle [1773] a method had existed for the extraction of impure protein samples from the fresh leaves of plants, very little extraction work was done until Osborne & Wakeman [1920] macerated spinach leaves and obtained protein from the juice. Since 1920 most of the work has been done by Chibnall and co-workers, with progressive improvements in technique: Chibnall & Schryver [1921]; Chibnall [1922; 1923; 1924]; Chibnall & Grover [1926]; Miller & Chibnall [1932]; and Chibnall *et al.* [1933].

By repeated maceration of spinach leaves in the presence of water Osborne & Wakeman [1920] extracted all but 16% of the nitrogen. They centrifuged the green juice to remove cell debris and flocculated two fractions of protein by adding successive portions of alcohol, the fractions, after purification by extraction with solvents, containing 12.9% N and 14.9% N respectively. Subsequently, Osborne *et al.* [1921] fractionated the nitrogenous constituents of lucerne leaves by macerating and extracting with various solvents, and obtained fairly pure specimens of protein by filtering the extracts through paper pulp before flocculating. Of the 56% of the leaf N which was not obtained in aqueous solution very little could be extracted by 93% ethyl alcohol or 0.075 N NaOH solution, but nearly 40% was extracted by 0.075 N NaOH in 60% alcohol. They noticed that the chloroplast material in the aqueous extract was removed by filtration. Considerable protein was flocculated from the filtrates of the aqueous and alkaline water-alcohol extracts.

The attention of Chibnall and co-workers has frequently been directed towards the preparation of protein samples in as high a state of purity as possible. Chibnall [1923] observed that the readily diffusible constituents of the leaf cells could be removed very well by plasmolysing with ether and other anaesthetics and pressing the leaves. Subsequently ether-water and "used" ether-water (i.e. ether-water already used once for the purpose) were substituted for ether, because they permitted better extraction of the protein when the leaves were macerated later in the presence of water. The juices, thus freed in advance from most of the diffusible solutes, were filtered through paper-pulp and the proteins were flocculated, coagulated and purified by extraction with various organic solvents. The yields have varied greatly (from 1 or 2 to 40% of the leaf protein), but some fairly "pure" preparations have been made. Using ether as plasmolysing agent

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preparations have been obtained from spinach and broad-bean leaves containing 15.95 % N and 1.7 % ash, and 16.05 % N and 4.3 % ash, respectively. These are the highest N contents of any preparations yet obtained from leaves.

With the object of improving the purity of preparations Osborne & Wakeman [1920], Chibnall & Schryver [1921] and Davies [1926] dissolved them in alkali and reflocculated with acid, and were usually successful in increasing the percentages of N. Foreman [1938] obtained small yields of relatively pure preparations by special techniques which did not involve solution in alkali.

There have been two aims in most of this protein extraction work: (1) to obtain large yields, in the belief that high-yield preparations are likely to be more representative of the whole protein in the leaves, and (2) to obtain "pure" preparations because of the difficulties and uncertainties which may attend the analyses of impure preparations. But unfortunately the two aims have usually proved to be mutually exclusive, and it has long been desirable to have some means of checking the representativeness of extracted protein samples. The only accessible means of classifying them and comparing them with the whole leaf-protein of which they represent a part and with the residual leaf-protein (i.e. that not extracted), is through their compositions. An extracted protein preparation may contain some 15 % N whereas, after removal of non-protein N, the whole leaf and the residue may contain only 3 or 4 % N. The methods of analysis employed must therefore be free from interference by very large amounts of impurities, and there are not, as yet, many such methods. Miller [1935] estimated the lysine, arginine and histidine contents of whole and extracted leaf-proteins of cocksfoot grass, and believed that the differences encountered, though fairly large, were of uncertain significance. The object of the present work was to obtain information of the composition of whole leaf-proteins, and to test the representativeness of samples of protein extracted by methods already in use and by any others which suggested themselves.

All materials from which non-protein N has been removed, whether they represent the whole leaf, the extracted preparations or the leaf residues, will be referred to hereafter as "protein preparations" and will be differentiated where necessary by the self-explanatory prefixes "whole", "extracted" and "residual" and the corresponding abbreviations W, E and R.

EXPERIMENTS AND DISCUSSION

The preparations (general)

Details concerning the fresh leaf materials and the extraction of samples of protein are contained in Tables I and II. The contents of Table II are explained later in the text and in accompanying remarks. Table I shows in the order of the columns: the label of the protein preparation (exp. number, followed by abbreviation showing whether "whole", "extracted" or "residual" and sometimes other distinguishing signs): the leaf-material source and the date: the percentage dry weight of the leaf material: the percentage of total N (*t*), coagulable N (*c*) and protein N (*p*) in the dry leaf: abbreviations indicating the method of extracting the sample of protein (if an extracted preparation): the percentage of the leaf N (*t*, *c* and *p*) appearing in an extracted juice or leaf residue: the percentage of N in the dry protein preparation; and finally the percentage of ash in it after ignition in air at about 600°. Sometimes a few % more or less total N could be found in the juice plus residue than was calculated to have been present in the fresh leaf material used. As there was never any definite evidence of real loss of N

Table I

Prep.	Source and date	% dry wt.	% N in dry leaf (t, c, p)	Method of extraction	% extracted and residual N (t, c, p)	% N in prep.	% ash prep.
1W	<i>Dactylis glomerata</i> (cocksfoot grass)	29. ix. 36	6.81t, — 4.50p	—	—	7.53	3.2
1E	"	"	"	w, c, w, p, .f.	—	14.1	1.1
2W	"	7. vi. 37	4.05t, — 3.36p	—	—	5.61	5.6
2E(a)	"	"	"	w, c, w, p, .f.	—	13.05	1.2
2E(b)	"	"	"	d, fs, w, p, .f.	71.5t, — 61.1p	12.6	1.4
3W	<i>Lolium perenne</i> (perennial rye-grass)	9. vi. 37	4.19t, — 3.38p	—	—	4.93	23.2
3E(a)	"	"	"	w, c, w, p, .f.	—	13.55	1.4
3E(b)	"	"	"	d, fs, w, p, .f.	—	12.35	6.4
4W	"	29. xi. 37	4.20t, 3.14c, 3.00p	—	—	5.12	13.3
4E'	"	"	"	d, c, pH 7, p, .c.	45.1t, 29.4c, 27.8p	13.1	7.3
4E''	"	"	"	, , pH 10, p, .c.	4.1t, 3.2c, 3.1p	8.15	25.8
4R	"	"	"	—	50.8t, 63.7c, 62.7p	3.45	19.9
5W	<i>Beta cicla</i> var. (beet spinach)	3. xii. 37	4.17t, 3.17c, 3.02p	—	—	6.14	9.7
5E'	"	"	"	d, c, pH 7, p, .c.	46.9t, 30.7c, 29.4p	13.3	6.3
5E''	"	"	"	, , pH 10, p, .c.	4.6t, 3.1c, 2.9p	12.1	7.6
5R	"	"	"	—	48.5t, 60.4c, 58.9p	4.06	12.8
6W	<i>Festuca ovina</i> (sheep's fescue grass)	19. v. 38	3.14t, 2.52c, 2.41p	—	—	—	—
6	[See Table II]						
7W	<i>Lolium perenne</i> (perennial rye-grass)	8. vi. 38	4.43t, 3.60c, 3.40p	—	—	5.77	4.7
7E	"	"	"	d, c, pH 9, sc, .	56.2t, — 46.5p	12.5	3.7
7R	"	"	"	—	43.8t, — 48.5p	3.54	5.6
7E1	"	"	"	d, c, pH 9, sc, ae, c.	50.7t, — 46.1p	13.2	3.0
7E1	"	"	"	—	5.5t, — 4.6p	8.5	—
7E2	"	"	"	d, c, pH 9, sc, ae, f.	36.8t, — 30.2p	14.65	0.6
8W	<i>Dactylis glomerata</i> (cocksfoot grass)	4. x. 38	7.34t, 6.16c, 5.92p	—	—	8.91	3.9
8E1	"	"	"	d, f, pH 9, sc, .	92.1t, 86.7c, 85.9p	13.25	3.1
8E2	"	"	"	d, f, pH 9, sc, ae, .	92.1t, 94.1c, 94.1p	13.4	3.8
8R	"	"	"	—	7.9t, 8.8c, 8.4p	1.82	7.8
8E3	"	"	"	d, f, pH 9, sc, .f.	69.1t, 60.3c, 59.7p	14.2	0.6
8E4	"	"	"	d, f, pH 9, sc, ae, c.	87.5t, 89.0c, 88.8p	14.55	2.2
8RE4	"	"	"	—	4.6t, 5.2c, 4.7p	6.16	—
8E5	"	"	"	d, f, pH 9, sc, ae, .	65.6t, 62.5c, 60.7p	15.0	0.6

Table II

8 g. portions of fresh leaves of *Festuca ovina* (22.7% dry wt.; 3.14% total N, 2.52% coagulable N and 2.41% protein N in the dry leaves) were macerated in the end-runner mill for 5 min. with 50 ml. of (i) water; (ii) pH 7.2 sodium phosphate buffer containing 1.5 g. P per litre; (iii) and (iv) pH 9.2 sodium borate buffer containing 1.1 g. B per litre; and the mash was centrifuged for 2 min. at 100 × gravity. From moisture content of leaves and moisture loss during maceration (estimated by tests with standard acid), the final volume of liquid was calculated to be 55.0 ml. Each 5 ml. therefore should have contained 5.17 mg. total N, 4.15 mg. coagulable N and 3.98 mg. protein N if all the N had been completely and homogeneously dispersed into the liquid. Compare with data in column (2).

In (i), (ii) and (iii), the 5 ml. aliquots of juice (left at pH 6 if water had been used, otherwise adjusted with alkali to buffer pH value) were centrifuged for about 30 min. at 25,000 × gravity to separate the granule from the non-granule fractions, judged by transparency of, and absence of "greenness" in the liquid layer. See columns (3) and (4). The granules, when later extracted with a protein solvent, were stirred with about 5 ml. of the solvent and the emulsion was left for 10 min. before recentrifuging. See columns (5)–(16). Data in column (17) were obtained from mixed extracts of the granules as indicated. The solvents were variously: 5 ml. of the aqueous solvents, water, diluted pH 7.2 buffer, diluted pH 9.2 buffer, and 0.05 N NaOH; 4.5 ml. of aqueous solvent plus 0.5 ml. of ether; 2.5 ml. of solvent plus 2.5 ml. of alcohol; and 2.5 ml. of aqueous solvent plus 2.0 ml. of alcohol and 0.5 ml. of ether.

In (iv) the juice itself was treated with a mixture of 0.8 vol. of alcohol and 0.2 vol. of ether before centrifuging. The figures in parentheses above certain values indicate the percentages of N in the dried precipitates concerned. The granule N: non-granule N ratio in (i) is believed to have been too high from sedimentation of a little non-granule protein (denatured?) as a pale top layer.

Form of N (1)	Juice M mg. (2) (i)	Non-		Action of solvents upon granules																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																							
		gran. N mg. (3)	Gran. N mg. (4)	Water				pH 7.2 buffer				pH 9.2 buffer				pH 7.2 buffer				pH 9.2 buffer																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																							
		Sol. N mg. (5)	Insol. N mg. (6)	Sol. N mg. (7)	Insol. N mg. (8)	Sol. N mg. (9)	Insol. N mg. (10)	Sol. N mg. (11)	Insol. N mg. (12)	Sol. N mg. (13)	Insol. N mg. (14)	Sol. N mg. (15)	Insol. N mg. (16)	Sum of 5, 7 and 9 (17)																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																													
Total Coagulable	4.69	2.96	1.73	0.14	1.59	0.21	1.52	0.18	1.55	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

(as NH_3 , for example), the discrepancies were attributed to sampling errors. Values shown in the table have been adjusted as for perfect recovery of total N.

Of the abbreviations concerning the method of extracting protein from the leaves, *d* signifies direct maceration with the protein solvent, or *w*, maceration after removal of readily diffusible leaf-cell constituents by plasmolysis with "used" ether-water and pressure in the Chibnall *et al.* [1933] process. Following these abbreviations, *c* means that the material was coarsely macerated in a mincing-machine, *f*, that it was finely macerated in an end-runner mill, or *fs*, that it was finely macerated with 20% by weight of sand in the runner mill. Then *w* signifies that water, or "*pH* 7 (or 9 or 10)" that a buffer solution of the indicated *pH* value, was the protein solvent with which the leaves were macerated. Following these, *p* signifies that the mash was pressed in a cloth, or *sc*, that it was sieved and the liquid centrifuged for 2 min. in a field of $100\times$ gravity, to separate the juice from most of the cell debris. Then *ae* indicates that a cold mixture of 0.8 vol. alcohol and 0.2 vol. ether was stirred into the juice with cooling and the mixture allowed to stand for 15–30 min. Finally, *f* means that the juice was filtered through a pad of paper-pulp, or *c*, that it was centrifuged for 30 min. in a field of $500\times$ gravity, before the protein was flocculated. Blanks in the sequence of abbreviations indicate omitted steps. The extractions were done at room temperature (10–25°).

In general, a higher percentage dry weight of leaf in any one species reflects greater age of leaf; and, ages being similar, a higher percentage of N in the dry leaf reflects better manuring. Inasmuch as it is believed that the leaf proteins of the species would all contain some 17.0% N if they could be obtained pure, the actual values for the preparations afford some measure of purity, and, in conjunction with the percentages of ash, some measure of organic impurity. It is believed that nitrogenous impurities were absent from the protein preparations.

Methods of estimation

Nitrogen estimations were made by the Kjeldahl method. The terms "coagulable nitrogen" and "protein nitrogen" have been explained in earlier articles [Lugg, 1938, 2, 3] and apply in the present article to the original leaf materials, to the extracted juices containing protein and other nitrogenous substances, and to the leaf residues after extracting juices. Briefly, "protein N" means N not extracted by successive applications of boiling alcohol, dilute citric acid, alcohol and ether, to a material containing "coagulable N"; and (usually) "coagulable N" means N left in insoluble material after heating at about 90° in dilute acetic acid at about *pH* 4.5. In some instances, to be discussed, coagulation was performed by heating to about 70° in water-alcohol mixtures (which sometimes contained ether until it had been boiled off) at a *pH* corresponding with that of a mixture of aqueous acetate *pH* 4.5 buffer with 0.8 vol. alcohol, and therefore above 4.5 but on a different *pH* scale (see, e.g. Richardson [1934]). By coagulating in presence of 0.8 vol. alcohol it was normally found that the coagulable N and protein N were increased in the case of a juice, but in the case of a dried leaf material they were almost unaffected as also were the percentages of N and ash in the resulting whole protein preparation.

Amide, tyrosine and tryptophan, and cystine plus cysteine and methionine estimations were made with the protein preparations by the methods described in the articles [Lugg, 1938, 1, 2, 3], wherein it has been shown that the estimations of amide, tyrosine, tryptophan, cystine plus cysteine (by differential oxidation) and methionine (by differential oxidation) are not subject to fresh systematic errors when some carbohydrates are added to a protein. It was

assumed that they would be satisfactory in the presence of much cellulose and other cell-wall constituents; and, in fact, satisfactory tests of the procedures for estimating tyrosine and tryptophan were made with much cellulose present. It was found too that amide values obtained with the whole and residual protein preparations by varying the hydrolysis procedure differed from those obtained by the standard procedure in much the same way as did values with extracted protein preparations [Lugg, 1938, 3]. Estimations with whole and residual protein preparations were sometimes made on a larger scale than was customarily adopted with extracted protein preparations.

The random errors of estimation with whole and residual protein preparations are believed to have been not more than about 1.5 times as great as they were in the case of extracted protein preparations, and these last are believed not to have exceeded the figures indicated in the following general description of Table III,

Table III

Prep.	% N	% amide N	% tyr.-N.	% try.-N	% cyst.-N (d.o.)	% meth.-N (d.o.)	% cyst. + meth.-N (d.o.)	SO ₄ -S × 100/N (d.o.)	% cyst.-N (HI t)	% meth.-N (HI t)
1W	7.53	5.10	2.44	1.90	1.38	1.46	2.84	1.07	—	—
1E	14.1	5.26	2.34	1.82	1.68	1.49	3.17	0.40	1.50	1.30
2W	5.61	5.04	2.53	1.78	1.41	1.61	3.02	0.36	—	—
2E(a)	13.05	4.95	2.32	1.80	1.55	1.42	2.97	0.78	1.35	1.22
2E(b)	12.6	5.02	2.39	1.88	1.44	1.52	2.96	0.65	—	—
3W	4.93	5.26	2.48	1.82	1.41	1.48	2.89	0.39	—	—
3E(a)	13.55	4.73	2.34	1.73	1.69	1.58	3.27	0.66	1.54	1.50
3E(b)	12.35	5.15	2.44	2.09	1.57	1.53	3.10	0.25	—	—
4W	5.12	5.20	2.41	1.78	1.47	1.59	3.06	0.76	—	—
4E'	13.1	4.96	2.43	2.05	1.63	1.52	3.15	1.12	1.20	1.07
4E''	8.15	5.64	2.28	1.62	1.48	1.49	2.97	1.30	—	—
4R	3.45	5.15	2.44	1.54	1.51	1.58	3.09	1.02	—	—
5W	6.14	5.28	2.63	1.50	1.45	1.43	2.88	0.63	—	—
5E'	13.3	5.18	2.57	2.01	1.47	1.44	2.91	0.91	1.24	1.17
5E''	12.1	5.18	2.68	1.71	1.59	1.39	2.98	0.73	—	—
5R	4.06	4.97	2.79	1.35	1.53	1.42	2.95	0.94	—	—
7W	5.77	5.21	2.45	1.92	1.42	1.50	2.92	0.62	—	—
7E	12.5	5.27	2.42	1.97	1.40	1.60	3.00	0.43	1.21	1.41
7R	3.54	5.30	2.51	1.88	1.48	1.45	2.93	0.74	—	—
7E1	13.3	5.15	2.35	1.92	1.46	1.47	2.93	0.34	1.17	1.36
7E2	14.65	5.17	2.39	1.96	1.37	1.46	2.83	0.34	1.25	1.40

which contains the results of analyses. In the order of the columns are shown: the characteristic label of the preparation: the percentage of N in it (error 0.5 %, affecting all the other errors): the amide content (error 1.5 %): tyrosine content (error 2 %): tryptophan content (error 3 %): cystine plus cysteine content, by differential oxidation (error 4 %): methionine content, by differential oxidation (error 5 %): the sum of the last two values (error 2 %): sulphate-S as a percentage of the weight of the N in the preparation (error from 3 % for the higher to 20 % for the lower values): cystine plus cysteine content, by titration of HI digests (error 3 %): and finally the methionine content, by titration of HI digests (error 4 %). The values have been corrected for such losses as the pure, free amino-acids undergo in the analytical procedures involved. To facilitate comparison of the compositions by way of proving the non-identity or assessing the probability of identity of the protein moieties of preparations (whole, extracted and residual), the results have been reported on a nitrogen basis, namely, as percentages of the N in the preparations appearing as the particular chemical species (amide or amino-acid) estimated. Although they are believed not to have

contained nitrogenous impurity, the preparations may have contained N in groups (such as nucleic acid) conjugated with some of the protein. Values in the last three columns cannot be used in comparing compositions. Those in the last two are probably low, though the precise significance of the disparities between them and the values obtained by the highly unspecific differential oxidation procedure is uncertain, whilst those in the previous column (sulphate-S) are to be regarded as measures of impurity.

(1) *Extraction test with Dactylis glomerata*

A check was made of the representativeness of protein extracted from a batch of *Dactylis glomerata* leaves by the method of Chibnall *et al.* [1933]. Details concerning the fresh leaf material (which was young and taken from a well-manured plot), and the whole and extracted protein preparations, are contained in Table I under the designations 1W and 1E. The results of partial analyses of the preparations are shown in Table III under the same designations. The S distributions were significantly different, the cystine plus cysteine content of 1E being considerably greater than that of 1W.

(2, 3) *Extraction tests with Dactylis glomerata and Lolium perenne*

The object of making the preparations 2W, 2E (a) and 2E (b) from leaves of *Dactylis glomerata*, and 3W, 3E (a) and 3E (b) from leaves of *Lolium perenne*, was to test again the representativeness of protein extracted by the method used in experiment 1 (giving now 2E (a) and 3E (a)) and further, to test the efficacy of a direct fine-maceration method which yielded 2E (b) and 3E (b). The surfaces of the *Lolium perenne* leaves were contaminated with soil. Details concerning the leaf materials and the extraction work are recorded in Table I, and analyses of the preparations in Table III. 2E (a) was of distinctly lower tyrosine content than 2W. Of 3E (a) and 3E (b), the former contained larger amounts of the sulphur-containing amino-acids and less amide than 3W, whilst the latter differed from 3W most noticeably in possessing a higher tryptophan content. Clearly, no significant improvement in the representativeness of extracted protein was achieved by macerating the leaves very finely; and, despite extensive extraction, the leaf residues having been macerated repeatedly with more water, considerable amounts of the leaf proteins were missing from the extracted preparations.

(4, 5) *Extraction tests with Lolium perenne and Beta cicla*

The unsatisfactory recoveries of protein in the fine-maceration tests in Exps. 2 and 3 suggested that much protein was being retained by the filter pads, possibly in association with the plastids and nuclei. In Exps. 4 and 5 (Table I) with leaves of *Lolium perenne* and *Beta cicla* respectively, it was accordingly decided to centrifuge, instead of filter, the juices which had been pressed through a cloth. The juices were centrifuged for 30 min. in a field of $500\times$ gravity, and although considerable chloroplast and other material was thus removed with cell-wall debris, the juices were still opalescent and strongly green in colour. A direct, coarse-maceration procedure was used in both experiments, and the initial extracting solvent was a pH 7 phosphate buffer containing 1.5 g. P per litre, providing preparations 4E' and 5E' respectively. The residues were then extracted with a pH 10 borate buffer containing 1.1 g. B per litre, but without further maceration, and yielded 4E'' and 5E'' respectively. It was hoped that

such nuclei, chloroplasts etc. as had been liberated from the cells but remained entangled with the leaf residues after the first extraction, would yield at least some of their protein to a pH 10 buffer. The high ash contents of the whole (4W and 5W), the extracted (4E' and 4E'', and 5E' and 5E'') and the residual (4R and 5R) protein preparations, are attributable to the fact that the leaf surfaces were contaminated with loamy soil.

The partial analyses of the preparations are recorded in Table III. They show that the proteins extracted at pH 10 differed considerably from those extracted at pH 7, and that the total extracted proteins were far from being representative of the whole proteins of the leaves. In neither case was the extracted plus residual protein N as great as the whole protein N. The losses were at first attributed to proteolytic activity in the extracts, but it was found later that the amounts of protein left in aqueous solution at pH 4.5, even after heating to 90°, are usually by no means negligible.

The one satisfactory aspect of these two experiments, 4 and 5, was that after making rough corrections for protein losses, all of any particular chemical species estimated in the whole proteins could be accounted for in the extracted and residual proteins to within the random errors of analysis.

(6) *Extraction tests with Festuca ovina*

A closer examination of the natures and reactions of the morphological units of the leaf cells was demanded by the findings of this earlier work, and a series of experiments was designed accordingly.

The proteins of plants are almost entirely intracellular, and in living, somatic cells appear to occur mostly in the protoplasm: only in ripening and drying seeds has much protein been found in the vacuoles. The protoplasmic protein is distributed between the nuclei, the ground mass of the cytoplasm and the more solid particles such as the plastids and mitochondria (or chondriosomes). Crystalline and amorphous protein particles may be present free in the vacuoles and cytoplasm, and embedded in the nuclei and plastids. The possibility cannot be ignored that the protein or protéins coming into each of these categories may differ from those in any other, and there may be differences as between one cell and another. Menke [1938], extending the work of Noack [1927], has shown that nuclei and chloroplasts may be separated from the rest of the protoplasm fairly readily by centrifuging and by other means; and Fauré-Fremiet [1913] found that mitochondria could be removed by centrifuging. The protoplasm is thus to be regarded as consisting of fluid and more solid fractions but no simple embracing terms are in use in reference to the entire, mixed cell contents. For simplification of subsequent descriptions and discussions, the terms "non-granule" will be applied to the mixed liquid fractions of vacuoles and protoplasm, and "granule" to the nuclei, plastids, mitochondria, membranes etc.

As a solvent for the protein contained in chloroplast material which had been extracted with alcohol and ether, Menke [1938] employed a reagent used by Osborne *et al.* [1921] and later by Davies [1926], namely, 0.075 N NaOH in 60% alcohol-40% water solution. It is probable that the nitrogen extracted from leaf residues by the earlier workers with this reagent was largely of chloroplast origin. Menke [1938] sometimes used dilute aqueous NaOH solutions as solvents for leaf proteins, and they have been used also by Kiesel *et al.* [1934] and by earlier workers. These strongly alkaline reagents, however, could scarcely be expected not to degrade any protein dissolved in them (see e.g. Blish & Sandstedt [1929-1930] and Neglia *et al.* [1938]).

In the earlier experiments with *Festuca ovina*, it was found that by macerating the leaves very finely in a runner-mill with water, or better with a pH 7 or 9 buffer solution, and then centrifuging the mash for a short time in low-intensity fields, the forms of leaf N (total, coagulable and protein) were distributed through the fluid in macro-uniformity and almost in the proportions occurring in the leaves, and very little unextracted N remained in the particles of cell debris which, together with some of the fluid, formed the lower layer of loose sludge. It was otherwise if any attempt were made to separate the debris by pressing the mash in a cloth, for the debris itself acted as a filter to retain preferentially some of the coagulable and protein N, an effect which could be correlated with a visible retention of chloroplast material. From the granules separated by centrifuging in high-intensity fields, it was found possible to extract protein by emulsifying with quite mildly alkaline buffers and adding ether, alcohol and alcohol-ether mixtures; but if ether were ever present in such quantity as to permit a separation of liquid layers the chloroplast material was largely carried up with the ether and the protein in it rendered rather insoluble in the buffer, presumably by denaturation. Furthermore, if very large proportions of alcohol were added the buffer solutions failed to be satisfactory protein solvents.

Typical later experiments with *Festuca ovina* leaves are described in Table II with its accompanying explanation. Of particular interest is the fact that the alcohol-ether-pH 9.2 buffer mixture proved to be almost as effective in dissolving protein from the granules as was the alcohol-NaOH solution mixture. It disintegrated the granules (or at least the chloroplasts), the residues of which retained almost all their chlorophyll and apparently most of their lipin and centrifuged down much more cohesively than undisintegrated granules do. Microscopically, the disintegration was correlated with a tendency of the chloroplasts to lose their shape, to stick together and to smear on glass. From suspension in the buffers themselves, the denser nuclei centrifuged down as a small cream-coloured layer beneath the centrifuged chloroplasts, very much as they did from suspension in water [cf. Menke, 1938], but when the lipid solvents were included with the buffers and the disintegration was pronounced, the cream-coloured layer could not be distinguished. The nuclei and mitochondria may have suffered a fate similar to that of the chloroplasts or they may have been carried down almost intact with the disintegrated chloroplasts. Some protein was obstinately retained by the disintegrated granule residues and it is not known to what morphological units it belonged, though it would probably have included any denatured protein, such as might have been present in dead cells.

Inasmuch as some N could be extracted from the granules without the aid of lipid solvents, it would seem that they might yield much of their protein to water or to plain mildly alkaline buffer solutions if they could be disrupted mechanically to a sufficient degree. It must have occurred to some extent during the grinding operations which yielded the juices examined.

As to the nature of leaf-cell nucleoproteins, the work of Belozersky & Chigirev [1936] and Belozersky & Dubrovskaya [1936] suggests that the protein parts proper are unlikely to be of strongly basic character like the protamines and histones.

Exp. 6 (iv) showed that the granule disintegration could be carried out in the presence of the rest of the protoplasmic and vacuole material. From a comparison of the N contents of the relevant protein micro-preparations, it seemed too that the lipid solvents could change the state of hydration or aggregation of certain substances in the juice (mucilages?) in such a way that they could be removed in part with the disintegrated granules by centrifuging in high-intensity fields, thus

reducing the extent to which they were able later to contaminate the flocculated protein. It will be noted that the proteins were more completely flocculated and coagulated from their solutions if alcohol was present, some of the extra "protein" obtained possibly being of proteose or similar nature.

(7) *Large-scale coarse-maceration extraction of Lolium perenne*

The presence of a mildly alkaline buffer during maceration of the leaves is favoured by the considerations that a fairly satisfactory solvent for solid (deposit) protein is thereby provided, that the other units comprising the granule fraction of the cell contents appear to suspend or emulsify in it better than they do in more neutral media, and that leaves which have so strongly acid a cell-sap that they fail to yield even their non-granule protein in solution when macerated with water might be expected to behave towards the buffer much as the *Festuca ovina* leaves behaved (cf. Chibnall & Grover [1926], who used dilute NaOH solutions to extract such leaves). In regard to the alkalinity of the buffer, the reversible-dissociation ranges of the proteins concerned should not be exceeded. It might be safe to use buffers at pH 10 or even pH 11. At all events, such evidence as does exist suggests that irreversible dissociation does not normally occur with proteins in the neighbourhood of pH 9, in which region the degree of reversible dissociation is frequently negligible (see e.g. Svedberg & Sjögren [1930, 1, 2]; Sjögren & Svedberg [1930, 1, 2]; and a recent review by Svedberg [1937]). Subsequent addition of lipid solvents would decrease the ionization constants of buffer acids (thereby normally increasing the pH, but on a different pH scale) and the dissociation and ionization constants of the proteins would probably be modified too, but the resultant effects in regard to protein dissociation are not known.

The above considerations apply whether the leaf material is macerated finely or coarsely. By repeated fine maceration and avoidance of any loss of the granule fraction it would seem from experiment 6 that almost all the protein could be extracted and obtained as a preparation which might not be very pure but must be almost perfectly representative, and that at some sacrifice of representativeness purer preparations could be obtained with the aid of lipid solvents. The large-scale extraction of leaf-protein samples, however, can be effected more easily by coarse than by fine maceration procedure. Many of the leaf cells are not ruptured by coarse maceration and those that are ruptured may not yield their contents to the juice in proper proportion if the maceration procedure itself involves a partial filtration by extrusion of the contents through split cell-walls. In principle, therefore, coarse maceration should amount to random clean cutting or tearing, without compression, and the requirements are fulfilled to a degree by a mincing-machine though not perfectly. *Lolium perenne* was one grass from which it had been lamentably easy to extract unrepresentative protein and it was accordingly chosen to provide fairly rigorous tests of procedures based upon the findings of Exp. 6 and adapted to large-scale extraction with the aid of the mincing-machine.

The leaf material was of medium age and had been grown in a rather shady environment. By finely macerating a small portion with a pH 9 buffer and centrifuging the juice as in Exp. 6, 32.8% of the leaf total N was found to be associated with the granule fraction containing all the visible chlorophyll. The main portion was passed through the mincing machine with three times its weight of a pH 9.2 sodium borate buffer containing 1.1 g. B per litre, the juice was separated from cell debris by violent shaking and stirring on a wire sieve (holes 1 mm. sq.) followed by centrifuging for 2 min. in a field of 100 × gravity,

and the residues were washed by stirring up with more buffer. The mixed juices were adjusted to pH 9.2 and divided up to give preparations 7E, 7E1, 7E2 and 7RE1 in Table I, 7RE1 representing disintegrated granule residue separated by the centrifuge before flocculating 7E1. The data have been calculated back as if all the juice had been used for each preparation. It should be noted that 7R, 7E1 and 7RE1 together contained virtually all the protein N calculated to have been present in the fresh leaves. 7E not having been flocculated and coagulated in presence of alcohol, some protein N (calculable from 7E1 and 7RE1 as about 10%) remained in solution in the extract, and gives the impression that much more non-coagulable N in proportion to coagulable N than was actually the case was extracted from the leaves. So too in the earlier extractions (Exps. 1, 2, 3, 4. and 5) must the proportions have been rather less unfavourable than they seemed. In the case of 7E2 there was considerable retention of protein N (and coagulable N) by the filter-pad but not much retention of non-coagulable N. It is one of the purest preparations which have been made from leaves of this grass. Whilst 7E might have been expected to contain too little of the non-granule protein, the reverse might have been expected of 7E1.

The whole, extracted and residual protein preparations (except 7RE1) were analysed, and the results are reported in Table III under 7W, 7E, 7E1, 7E2 and 7R. They showed, to within the limits of the random errors of analyses, no evidence of unrepresentative extraction of protein in any of the three preparations obtained: a most gratifying result in view of the hypothetical difficulties discussed. Apparently the large amount of protein retained by the filter-pad in the preparation of 7E2 was itself moderately representative of the whole protein.

(8) *Small-scale fine-maceration extraction of Dactylis glomerata*

Experiments were made with *Dactylis glomerata* with the object of generalizing further, if possible, some of the findings made with *Festuca ovina* (6) and *Lolium perenne* (7), and at the same time of obtaining high-efficiency extraction of the protein on a larger scale than in Exp. 6. 100 g. of fresh leaves of young-medium age, estimated by the method used in Exps. 6 and 7 to contain 33.1% of the total N in the granule fraction, were macerated in several portions in the runner-mill with a pH 9.2 borate buffer and the juice was separated from the residues with the aid of the sieve and the centrifuge as in Exp. 7, the residues being treated similarly twice in succession. The mixed juices, occupying a volume of nearly 1 litre, were adjusted to pH 9.2 and divided up for the preparation of 8E1, 8E2, 8E3, 8E4 (with 8RE4) and 8E5 as shown in Table I, the data being calculated back as if the entire amount of juice had been used for each preparation. 8RE4 represents the disintegrated granule material separated by the centrifuge before flocculating 8E4.

In general the results confirmed those found with the other plant species in Exps. 6 and 7. Again it was seen that the flocculation and coagulation of the protein in the juice were assisted by the presence of alcohol. In fact the recoveries of the whole (8W) coagulable N and protein N between the extracted and residual material, 8E2 and 8R or 8E4, 8RE4 and 8R, were a little in excess of 100%, and suggested that in drying the leaf material at 80° some 2% of the protein N had been converted into non-coagulable N. Just as in Exp. 6, centrifuging in a field of 500 × gravity (8E4) was less effective in preferentially removing contaminating substances from the juice containing alcohol and ether than filtration (8E5) was, but a field of 25,000 × gravity might have been as effective had it been possible to employ the high-speed centrifuge with such quantities of juice. Much protein was retained by the filter-pads whether alcohol and ether were

present (8E5) or not (8E3), but the former preparation, which appears to be the purest yet made from leaves of this grass, was considerably purer than the latter and was, presumably, more nearly representative of the whole leaf-protein. 8E2 must have been highly representative, whilst 8E1 may have been slightly deficient in the non-granule fraction. Of the total N in the residue 8R, rather too small a fraction in comparison with that of the original leaf material, was non-coagulable, suggesting that the residue contained insufficient non-granule protein to be representative of the whole. On the other hand it contained only 8.4% of the original protein N.

It is presumed that preparations similar to 8E4 and 8E5 could have been obtained by macerating the leaves in the presence of the lipid solvents, but the general effects of having lipid solvents present at this stage were not investigated.

Further considerations

Local acidity, causing differential flocculation of some of the non-granule proteins, is the only obvious hypothesis to be advanced in explanation of the rather pronounced differences in composition between 3E(a) and 3E(b). In passing it should be mentioned too that at pH values below 6 the non-granule proteins are usually very prone to surface denaturation. In the absence of salt their solutions in dilute HCl on the acid side of the isoelectric regions may frequently be boiled without appreciable coagulation.

It is of interest to compare the partial compositions of the whole proteins of the three batches of *Lolium perenne* leaves (3W, 4W and 7W), and those of two batches of *Dactylis glomerata* leaves (1W and 2W). The differences are small and of the most doubtful significance. In the first place, age of the leaves and climatic and manurial conditions and locality of growth appear to have been without appreciable effect upon composition of the whole leaf-proteins. In the second place there is no evidence that the whole leaf-proteins of the two species from the order *Gramineae* differed in composition. The whole leaf-protein of the single batch of *Beta cicla* from the order *Chenopodiaceae*, on the other hand, differed from these in having a lower tryptophan and a higher tyrosine content.

Even when appropriate precautions were not taken during extraction and the extracted proteins were demonstrably unrepresentative of the whole leaf-proteins, the differences in composition were not very great. Previously reported partial compositions of extracted protein preparations [Lugg, 1938, 2, 3] are therefore unlikely to have been seriously different from those of the whole proteins of the leaves from which they were obtained.

SUMMARY

Samples of protein extracted from plant leaves in various ways have been examined for representativeness. Points favouring the use of mildly alkaline buffers, when macerating leaves with the object of dispersing the protein-containing cell-units into the juice, and the difficulties of avoiding preferential loss of the proteins belonging to the "granular" units (nuclei, plastids, mitochondria etc.) when subsequently removing cell-wall material and other impurities, have been examined and discussed. It has been shown that the addition of lipid solvents, such as alcohol and ether, to the mildly alkaline juice permits most of the protein of the granule fraction to pass into solution; and the use of such solvents in the preparation of purer samples of protein without serious sacrifice of representativeness, has been demonstrated. Incidentally, the proteins

are much more completely flocculated by acid in their isoelectric regions and coagulated by heat, if alcohol is present in the solutions containing them.

The amide, tyrosine and tryptophan contents and the S distributions (cystine plus cysteine and methionine contents) of the whole proteins of leaves, provided no evidence of variation in composition with the age of the leaves or the manurial and climatic conditions or locality of growth; but composition may vary with plant species.

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