CCLVI. THE PROTEINS OF GRASSES.
II. A NEW METHOD OF PREPARATION.

By ALBERT CHARLES CHIBNALL, ERNEST JOHN MILLER, DONALD HUGH HALL and ROLAND GORDON WESTALL.

From the Biochemical Department, Imperial College of Science and Technology, South Kensington, London.

(*Received November 1st, 1933.*)

In the preliminary paper of this series [Miller and Chibnall, 1932], it was pointed out that the original ether method of Chibnall [1923] for the preparation of leaf proteins gave negligible amounts of protein when applied to various grasses, and a modification involving the use of ether-water in lieu of ether was described which in certain cases had enabled us to prepare the protein from cocksfoot. The yield of protein obtained however varied greatly and in most cases was disappointingly small. Many experiments were made to find out the inherent weakness of the method employed, and one of them (Exp. 4) described in the preliminary paper suggested that the ultimate yield of protein could be enhanced two- or three-fold if the grass, instead of being treated as soon as possible after cutting, was first of all set aside for about 8 hours, as though some change affecting the "aggregation" of the leaf proteins took place during this short period, thereby enabling them to disperse more readily into colloidal solution at the appropriate stage in the preparation. At the same time it was clearly pointed out that the influence of ether or ether-water, together with a "time factor," on the cytolysis of the leaf cell protoplasts—the first and essential step in the preparation of the leaf proteins—was not yet clearly understood, for although the use of ether-water and a "time factor" of 8 hours gave fair yields of protein from grasses cut in spring, they gave, curiously enough as it seemed at the time, negligible yields of protein from grasses cut from the same plots the following autumn.

Further experiments carried out during the last 18 months have indeed shown that our former conclusions were quite erroneous. Ether-water has been used as a cytolysing agent in the preparation of proteins in excellent yield from a large number of grasses and other forage crops, and we now know that the essential point in its employment is that it must have been used at least once before to cytolyse an appropriate amount of grass. This subtle difference in behaviour between what we shall henceforth refer to for convenience as "fresh" and "used" ether-water was quite unlooked for, because our previous experience in the use of ether itself as the cytolysing agent for leaves such as spinach or runner bean had taught us to look upon the ether content of the ether-water as the potent factor in its use. When fresh ether-water is used to cytolyse leaves it not only becomes contaminated with sap which exudes from the leaf cells but it also loses ether by evaporation during the subsequent handling of the leaf material. For this reason it had been customary in our laboratory to employ a new sample of ether-water with each portion of grass undergoing treatment. Occasionally, however, a shortage of ether compelled us to use the ether-water a

second or third time, and it is a coincidence that this occurred when a sample of the grass had been deliberately set aside for 8 hours. The greatly increased yield of protein then obtained was naturally, but we now know wrongly, ascribed to the "time factor" mentioned above. Further investigation very soon disclosed the nature of the extraordinary difference in action between "fresh" and "used" ether-water and since we now believe that this slight modification in technique permits the preparation of proteins in good yield not only from leaves of herbaceous plants which were amenable to the earlier ether treatment but also from leaves of ligneous plants from which it has hitherto been impossible to prepare proteins at all, it is necessary to describe the cytolysing action in some detail.

When cut grass is immersed in "fresh" ether-water for ten minutes the latter slowly turns brown. The leaf cells are cytolysed and the protoplasts become freely permeable to the aqueous contents of the vacuoles; consequently those constituents of the cell whose molecules are sufficiently small will diffuse freely through the cellulose wall into the ether-water. If a section of this cytolysed grass be cut the cells present the same appearances as those of spinach leaves cytolysed with ether [Chibnall, 1923]. In each cell the protoplast has collapsed and has shrunk to one end, the rest of the cell being filled with a brown water-clear liquid which is the vacuole fluid diluted with ether-water.

If a fresh batch of cut grass is now immersed in this "used" ether-water, cytolysis of the cells takes place to all appearances as readily as before, and the ether-water becomes further contaminated and more darkly coloured. Two striking differences can, however, be readily observed. In the first place there is a relatively enormous uptake of "used" ether-water by the cells, presumably before the semi-permeability of the protoplasts has been completely destroyed. In the second place a cut section shows that there is no evidence of collapse or shrinkage of the protoplasts, although these have been rendered freely permeable. The section, in fact, differs but little from that of a fresh untreated blade of grass, in which the turgid protoplasts fill the whole interior of the cell.

It is not possible at the present time to give a physiological explanation of the difference in behaviour between the "fresh" and "used" ether-water, but we believe that the following account affords a reasonable explanation of certain of the observed facts which bear on the yield of protein ultimately obtained. When the cut grass is immersed in "fresh" ether-water, the semi-permeability of the protoplast is destroyed extremely rapidly, so that the fluid of the vacuole, which contains a high concentration of solutes, is suddenly released. This fluid, as it diffuses out through the now freely permeable protoplast, causes partial dehydration or perhaps partial denaturation of the colloidal proteins which are one of the chief components of the cytoplasm, and may thus help to bring about the collapse and shrinkage of the protoplast. At the stage in the preparation of the proteins therefore when the pressed and washed leaf residues are ground up with water the cytoplasmic contents of the cells are not readily dispersed into colloidal solution. When "used" ether-water is employed the cytolysis of the cells is brought about in a modified way. The anaesthetic action of the ether is depressed, so that instead of causing the almost instantaneous death of the cell there is in the earlier stage only a slow decrease in semi-permeability. In some way which it is not yet possible to explain the response of the cells is changed, with the surprising result that water passes rapidly through the protoplast into the vacuole. The ether anaesthesia ultimately renders the protoplast freely permeable, but the vacuole fluid has been by now so diluted with ether-water that when it diffuses out through the protoplast the colloidal proteins suffer no

appreciable dehydration or denaturation, so that not only is the protoplast left, to external appearances, unchanged, but at the stage in the preparation of the leaf proteins referred to above the cytoplasmic contents of the cells are readily dispersed into colloidal solution. Appropriate experiments have shown that this modified action of "used" ether-water is due not to the lowered concentration of ether but to the substances which diffuse out from the cytolysed cells, and ether-water which has been used initially with one particular species of grass will act as "used" ether-water for any other species.

If one accepts the validity of the above explanation, it appears that the high yields of protein obtained from spinach and other leaves by the older ether-method, which caused the collapse and shrinkage of the protoplasts, were due to the much lower concentration of solutes initially present in these leaf cell vacuoles.

Experimental.

Materials used. The grasses used in the present research were pure strains taken from specially cultivated plots which were sown in the autumn of 1931.

Table I. *Details of proteins prepared from various grasses.*

Species	Date of sampling	Sample of leaf material: Fresh weight kg.	Dry weight %	Total N %	Total protein-N %	Extracted protein: Total weight g.	N (Ash-free) %	Yield in % of total leaf protein
Cocksfoot (*Dactylis glomerata*) (Batch Q)	20. v. 32	8·0	14·5	6·4	5·3	73	14·1	16·8
" " (" R)	27. v. 32	17·0	14·1	6·5	5·0	271	13·6	30·9
" " (" S)	3. vi. 32	20·0	13·0	6·8	5·7	245	14·0	23·3
" " (" U)	16. vi. 32	16·0	17·5	5·8	5·1	216	13·0	19·8
" " (" AB)	29. v. 33	30·0	13·0	6·15	5·4	397	14·6	27·5
" (" AC)	7. vi. 33	16·0	15·0	6·0	5·3	210	13·4	22·4
" " (" AD)	22. vi. 33	30·6	17·8	5·65	4·9	360	13·2	17·9
" " (" AE)	11. ix. 33	7·5	18·3	6·07	5·5	97	13·3	17·5
Rough-stalked meadow grass (*Poa trivialis*)	4. x. 32	2·9	18·0	5·6	4·9	26	13·4	13·6
" "	27. vi. 33	12·0	14·0	6·2	5·3	89	13·8	13·7
Timothy (*Phleum pratense*)	18. x. 32	0·9	27·0	4·7	4·1	7	13·8	9·7
Chewings fescue (*Festuca rubra* var. *fallax* (Hack))	26. ix. 32	7·5	16·0	6·15	4·8	73	14·1	17·8
Hard fescue (*Festuca duriuscula*)	29. ix. 32	4·0	22·5	6·80	5·5	37	15·0	11·1
" "	29. vi. 33	8·0	19·0	5·95	4·9	65	14·6	12·8
Red fescue (*Festuca rubra*)	29. ix. 32	2·0	21·0	6·15	5·0	27	14·4	18·4
" "	29. vi. 32	8·0	16·0	5·85	4·8	77	14·2	17·7
Tall fescue (*Festuca elatior*)	11. x. 32	1·4	16·6	5·75	5·0	13	13·7	15·4
" "	26. vi. 33	4·0	17·0	5·8	4·9	40	13·6	16·3
Meadow fescue (*Festuca pratensis*)	18. x. 32	0·7	32·0	3·4	2·9	7	13·8	14·7
" "	27. vi. 33	6·0	15·0	5·7	4·8	(6)	13·9	—
Italian ryegrass (*Lolium italicum*)	11. x. 32	1·7	13·7	6·3	5·3	13	14·0	14·8
" "	26. vi. 33	4·0	15·0	5·9	5·2	45	14·1	20·5
Perennial ryegrass (*Lolium perenne*)	6. xi. 33	7·0	18·1	4·6	3·8	82	12·8	21·8
Crested dog's tail (*Cyanosurus cristatus*)	18. x. 32	1·1	25·3	4·5	3·7	14	13·8	18·6
" "	27. vi. 33	6·0	16·0	5·25	4·5	46	14·9	16·1
*Bent (*Agrostis* sp.)	25. x. 33	4·0	27·0	2·43	2·1	32	10·3	14·6
*Yorkshire fog (*Holcus lanatus*)	23. x. 33	2·3	20·2	3·26	—	4·1	11·8	—
*Wild white clover (*Trifolium repens*)	18. x. 33	26·4	13·6	5·09	4·3	319	13·2	28·0
*Red clover (*Trifolium pratense*)	23. x. 33	9·3	23·7	2·71	2·4	101	12·8	24·5
*Lucerne (*Medicago sativa*)	29. vi. 33	12·0	21·0	3·25	2·8	61	14·4	12·6
*Yarrow (*Achillea Millefolium*)	24. x. 33	10·0	10·0	3·92	3·4	26	10·0	7·6

* From plots which had not received a dressing of ammonium sulphate.

In the spring of 1932 and 1933 each plot received a heavy dressing of complete fertiliser, and to obtain the maximum amount of protein from a given quantity of grass the plots were heavily treated at appropriate intervals with 3 cwt. of ammonium sulphate per acre. About 8–10 days before the grass material was required the plots were closely cut back with a mowing machine and then dressed with the fertiliser. In the absence of rain the plots were watered daily, and when cut with the mower to provide the experimental material the blades stood 3–5 inches high. In the case of cocksfoot, the protein of which we are using for amino-acid analysis and therefore require in large amount, a second cutting was taken about 7 days later. After growing for another week the plots were again cut back (grass discarded) and treated as before with ammonium sulphate, when they were again ready to give two high-nitrogen crops at the time intervals mentioned above. Details of the samples used are given in Table I. For convenience the total protein-N was determined by the conventional method of Stutzer. As one of the ultimate objects of the present research on grasses is to determine the nutritive value of forage crops we have also prepared proteins from lucerne, red and white clover and yarrow. All the proteins described in Table I were prepared by a standard method founded on the principles discussed above, and the practical details will be best illustrated by describing a typical preparation of cocksfoot protein in some detail.

Preparation of protein from 20 kg. of freshly cut cocksfoot.

Some beds of cocksfoot at the Imperial College Field Station at Slough were cut back with a mowing machine on May 20th, 1932, and then dressed with 3 cwt. of ammonium sulphate per acre. A first cutting (Batch R) was taken on May 27th and a second (Batch S), with which the present experiment is concerned, on June 3rd at 7 a.m. There was no appreciable dew, and the total fresh weight was 22 kg. the dry weight 13·0 % and the N 6·8 % of the dry weight.

A sample of 2 kg. was immersed in 5 litres of "fresh" ether-water contained in a deep enamelled pan, and at the end of 10 minutes the pan was tilted so that the resulting brown liquid could be drained off from the grass. The volume of "used" ether-water thus collected was 4500 cc., the remaining 500 cc. being retained on the surface of the grass. In many of the experiments not recorded in detail in this paper the batch of grass had been cut early in the morning following a rainy night. In such cases the surface of the grass was already wet, and when a sample was treated with "fresh" ether-water as described above there was no loss on draining, showing that the loss of 500 cc. in the present case was due to the "wetting" of the surface of the blades. The cytolysed grass was next enclosed in thick filter-cloth, which was placed in the steel cylinder 11 inches high and of 6 inches internal diameter belonging to a Buchner press. A well-fitting plunger was used to apply the maximum pressure for 4 minutes. The volume of expressed juice was 1400 cc. and on removal from the press the cylindrical cake of compressed grass was 9 cm. high. Nothing further was done with this sample, which had been worked up merely to provide the necessary "used" ether-water for the preparation of protein from the remaining 20 kg. of grass.

Another sample of 2 kg. was next immersed for 10 minutes in this 4500 cc. of "used" ether-water. On draining off the liquid only 3100 cc. was collected, so that if we assume that 500 cc. were required to "wet" the grass the remaining 900 cc. must have been actually taken up by the 2 kg. of grass. This enormous

intake of water by grass cytolysed with "used" ether-water has been commented on at some length above. The sample of grass was next enveloped in filter-cloth and pressed for 4 minutes as before. On removal from the press the cylindrical cake of grass residue did not remain compressed as in the previous sample, but the blades of grass separated slightly so that the height of the cake was 16 cm. as against 9 cm., again emphasising the fact that "used" ether-water had brought about cytolysis with less internal breakdown in the leaf-cells than was the case with "fresh" ether-water. The residue was allowed to imbibe water for 4 minutes and pressed as before. This operation was repeated twice more in order to wash away the easily diffusible contents of the cells. The final leaf residue was then ground to a pulp in a meat chopper with 3 litres of water, and the débris of cell wall material removed by squeezing through silk gauze. This débris was again treated in a similar way with a further 2 litres of water, and the two green colloidal extracts thus obtained were filtered with very slight suction on a 24 cm. Büchner funnel through a well-rammed pad of paper-pulp about 6–7 cm. thick. A clear brown protein filtrate was thus obtained.

Meanwhile a third 2 kg. sample of grass was immersed in the 3100 cc. of "used" ether-water given by the previous sample, 1900 cc. of press-juice being added to bring the total volume to 5000 cc. The remaining eight 2 kg. samples of grass were also treated in a similar way at such time intervals that there was always sufficient green colloidal extract to permit of continuous filtration through two of the paper-pulp pads. In each case the volume of "used" ether-water was made up to 5000 cc. with press-juice, and before the treatment of the 7th sample of grass it was reinforced by shaking with 100 cc. of ether.

The volume of the final filtrate was 47·3 litres, and 580 cc. of 2·04 *N* HCl were required to precipitate the protein at its isoelectric point. After standing overnight the supernatant liquid was syphoned off and the protein coagulated by heating on a water-bath. It filtered readily at the pump, and was purified by extracting once with boiling water, then boiling 5 times successively with 95 % alcohol and finally once with absolute alcohol. The weight of the moisture-free protein was 245 g., and it contained 13·9 % of N and 0·9 % of ash. The N, ash-free, was 14·0 %. As only 20 kg. of the batch of grass had been used to prepare the protein this yield represents 9·3 % of the total dry weight of the grass, 19·1 % of the total grass-N and 23·0 % of the total protein-N. These figures are twice as great as those obtained from spinach by the original ether method of preparation [Chibnall, 1924] and from cocksfoot in previous experiments with ether-water [Miller and Chibnall, 1932].

Discussion.

When the green colloidal extract referred to in the previous section was filtered through paper-pulp practically the whole of the protein passed through into the filtrate, very little being retained with the green fatty material on the paper-pad. We have always found this to be the case with the new modified ether-water method of preparation, whereas in the original experiments with ether [Chibnall and Grover, 1926] about one half of the protein was retained on the paper-pad. In former papers this fraction, which could not be readily separated from the fatty material, was referred to for convenience as the "combined" protein, while that which passed freely through the filter was referred to as the "soluble" protein. An extended research which has been made into the fatty materials present in leaves does not suggest that any true chemical combination can exist between the fats or phosphatides and the proteins, and we now believe that the "combined" protein previously obtained with ether or

"fresh" ether-water was simply a fraction of the "soluble" protein which had undergone partial dehydration or denaturation. It appears to us therefore that we have now reached a stage in the investigation of leaf proteins when such arbitrary distinctions as "combined" and "soluble" proteins have become unnecessary, and we have accordingly discontinued their use in this paper.

In comparing the yields of protein from various grasses given in Table I with that of spinach, the most successful preparation made by the old ether method, it is to be remembered that the object of the present research was to prepare the grass proteins as readily as possible in amount sufficient for analysis. The cells of spinach leaves have very thin walls and are readily disintegrated in a meat chopper or mill to give a maximum yield of protein. Blades of grass on the contrary have thick, more fibrous, cell walls, and the great labour involved in the grinding operations of a large scale preparation precludes any attempt being made to obtain maximum disintegration of the cells. Were this possible we believe that yields of 50 % or more of the total leaf protein could be readily obtained.

The protein of the grass residues is retained in unopened cells, and there seem to us no valid grounds for assuming that it differs in any way in composition from that which we obtain from the cells which have actually been torn open. We feel justified in claiming therefore that our protein preparations are representative of the whole protein of the leaf, and we shall interpret the results of our amino-acid analyses accordingly. But as we have repeatedly emphasised in previous papers there is as yet no evidence to show whether the preparations which we obtain from leaves consist of one particular protein, or whether they are mixtures of several proteins having similar physical properties.

The properties of the grass proteins are similar to those of the numerous other leaf proteins described by Chibnall and Grover [1926]. The impurity discussed at some length by Miller and Chibnall [1932] is present in all the new preparations; as a general rule we find that proteins with a high N content are obtained from young nitrogen-treated grass having a high protein content and low total dry weight, emphasising the view already expressed that the impurity is merely an adulterant with similar solubilities to those of the proteins.

Summary.

The ether-water method for preparing the proteins of leaves has been modified, and excellent yields of protein have been obtained from several pure strain grasses and certain forage crops.

The essential point is that the ether-water must have been used at least once before to cytolyse an appropriate amount of leaf material. This extraordinary difference in action between "fresh" and "used" ether water is discussed in some detail.

We should like to record our thanks to Prof. V. H. Blackman for many interesting discussions during the course of this work, to the Imperial Chemical Industries for a grant to cover the cost of this research, and to Mr G. E. Blackman of Jealott's Hill Research Station for the supply of pure-strain grasses.

REFERENCES.

Chibnall (1923). *J. Biol. Chem.* **55**, 333.
—— (1924). *J. Biol. Chem.* **61**, 303.
—— and Grover (1926). *Biochem. J.* **20**, 108.
Miller and Chibnall (1932). *Biochem. J.* **26**, 392.