LI. INVESTIGATION ON THE NITROGENOUS METABOLISM OF THE HIGHER PLANTS. PART V.

DIURNAL VARIATIONS IN THE PROTEIN NITROGEN OF RUNNER BEAN LEAVES.

By ALBERT CHARLES CHIBNALL.

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

(*Received February 21st, 1924.*)

THE object of the present research was to establish definitely whether there is a diurnal fall in the protein content of runner bean leaves at night.

In a former paper [Chibnall, 1923, 2] the author discussed at some length the available evidence concerning the diurnal changes in the total nitrogen content of foliage leaves, and concluded that there was undoubtedly a fall at night. In comparing the day and night samples of leaves three methods had been used to estimate the total nitrogen present, namely, those in which it was expressed as

A. Weight in terms of a certain number of leaves.

B. A percentage of the dry leaf weight.

C. A percentage of the fresh leaf weight.

It was further emphasised that method B might lead to an inaccurate and misleading interpretation of the diurnal change: that method A was unreliable unless some regard was paid to sampling errors: that method C would give the most accurate interpretation of the true diurnal change; conclusions amply justified by the results of the present research.

The above criticisms will apply with equal force to any determination concerning the diurnal variation in the protein nitrogen content of the leaf; in the following paragraphs the work of earlier investigators is discussed in terms of them.

Schulze and Schütz [1909] made a thorough investigation into the seasonal and diurnal changes in the leaves of the box elder (*Acer negundo*). The leaves were picked from two trees 15–20 years old, and those in each sample were chosen as far as possible of equal weight and leaf area. The evening leaves were picked between 6 p.m. and 6.30 p.m., those on the following morning between 5 a.m. and 6.30 a.m., depending on the season. The leaves were

rapidly dried at 90° (which may have brought about slight proteolysis), and protein nitrogen determined by Stutzer's method. They themselves interpreted their results by method A; it is possible from the data they supply to recalculate those by method C. The results by both methods are given in Table I.

Table I. *Showing the change in the amount of protein nitrogen in the leaves of* Acer negundo *at night, expressed as a percentage of the day value.* (*Schulze and Schütz.*)

Date picked	Change: Method A %	Change: Method C %
7 May	- 10·20	- 9·18
6 June	- 16·62	- 6·88
5 July	- 8·29	- 3·60
2 August	+ 2·51	+ 1·98
25 Sept.	+ 4·77	+ 4·73

In the absence of sampling errors the present author prefers to interpret the results in terms of method C, which clearly shows that there is a decided fall in the protein nitrogen at night during the period of physiological activity of the trees. Further, the figures for the water-soluble nitrogen, which remain more or less unchanged, show that the lost protein nitrogen has been translocated away from the leaf. When the leaves reach maturity, and growth has more or less ceased, the withdrawal is no longer apparent. The somewhat large rise in the protein nitrogen on the night of 25 Sept. may be a misleading interpretation, since the leaves had then already begun to fall, indicating more or less rapid loss of water due to dehydration.

Suzuki [1897] attempted to find the diurnal change in the leaves of several plants. He expressed his results in terms of method A, but did not take into account sampling errors, nor were sufficient data given for results by method C to be calculated. The diurnal changes occurring between 6 a.m. and 6 p.m. were compared. The leaves were air-dried at an unstated temperature, so that proteolysis was possible. Protein nitrogen was determined by Stutzer's method. His results are given in Table II, the figures in brackets after each plant denoting the number of leaves per sample used.

Table II. *Showing the change in the protein nitrogen in the leaves of various plants at night, expressed as a percentage of the day value.* (*Suzuki.*)

Date picked	Name of plant	Change by method A %
—	*Wistaria brachybotrys* (400)	- 16·6
17 Sept.	*Phaseolus mungo* (200)	- 9·9
29 Oct.	,, *vulgaris* (50)	- 10·9
5 June	*Solanum tuberosum* (220)	- 5·1
18 Sept.	*Batatus edulis* (100)	- 4·2
26 Sept.	*Polygonum fagopyrum* (450)	- 7·5

Suzuki gives no indication as to the physiological period at which his leaves were picked, but as most annuals show continuous growth until the death of

the plant, it is probable that there is a continuous call on the leaves for nitrogenous compounds. Though sampling errors were not taken into account Suzuki used in most cases a large number of leaves, so that his results, which are in general agreement with the better experimental data of Schulze and Schütz, are worthy of consideration.

Pigorini [1914] studied the diurnal variations in the leaves of the mulberry (*Morus alba* var. *Limoncina*). Thirty-eight leaves were picked at 5 p.m. 7 Sept. and 39 leaves at 5 a.m. the following morning. After drying, the protein nitrogen was determined by a modification of Stutzer's method. His result by method C shows a fall in the protein nitrogen content at night of 11·5 %, the major part of this lost nitrogen having been translocated away from the leaf.

The above results seem to indicate that during periods of physiological activity in the higher plants protein decomposition takes place in the leaf at night. The figures quoted above, however, must be accepted with caution, and in no case as quantitative, since the protein nitrogen has been estimated by Stutzer's method. This method is conventional only, and assumes that after the dried, ground up leaves are boiled with Stutzer's reagent, all the nitrogen remaining insoluble is to be regarded as protein nitrogen. Further, unless the drying of the leaves is very rapid, there will be loss of protein due to autolysis [Chibnall, 1922].

All the results quoted above suffer from the disadvantage that the diurnal change has been taken on one sample of day and night leaves only, so that errors due to sampling and individual differences are uncontrolled. In the present research nine diurnal samples of runner bean leaves have been taken, thereby allowing the degree of probability of the recorded results to be calculated.

General Experimental Methods.

Materials used. The plant used was the runner bean (Scarlet Champion), *Phaseolus vulgaris* var. *multiflorus*. The seeds were planted during the first week of April, 1923, in unmanured ground at the Physic Garden, Chelsea, following beans of similar species. Growth was delayed by cold weather in May, but by mid-August, when the experiments to be described in this paper were commenced, the plants were fully grown and in process of pod-formation.

Methods of sampling. The leaves of *P. vulgaris* var. *multiflorus* are pinnate trifoliate. Those used in the present experiments were fully grown, and were chosen from the upper parts of the plant, thereby ensuring that their surfaces were free from soil contamination.

A day or so before the experiment was to be performed the plants were carefully examined, and three batches each of about 100 leaves, which appeared to have basal and opposite leaflets of equal development, were selected. These were marked with coloured paint on the terminal leaflet to aid future identification.

At 8 p.m. on the night of the experiment, just before sunset, one of every

pair of basal and opposite leaflets was removed without any attached petiole. As the leaflets were picked they were placed one above the other in a closed box so as to reduce respiration to a minimum, and as soon as each batch was collected the total weight was taken. The leaflets were then counted. The time required for the picking and weighing was 20 minutes, so that all three batches could be dealt with in an hour.

At 6 a.m. the following morning, about an hour and a half after sunrise, the corresponding leaflets were detached and treated in a similar way.

Methods of manipulation (separation of vacuole material from the leaf cells). The ether method recently described by the author [Chibnall, 1923, 1] was used[1]. In that paper it was shown that the leaf cell constituents of spinach were separated into two portions, consisting of

(*a*) An extract containing *all* the vacuole content, with possibly some constituents of the cytoplasm, which, at the time of death, have either passed into the vacuole, or have been subsequently removed from the cytoplasm by the dilute acid (0·002 *N*) used for washing the residue.

(*b*) A residue consisting of cell wall material, together with the cell cytoplasm, from which some of the constituents may have been removed, as per (*a*) above. The cytoplasmic material could afterwards be extracted by grinding, and was found to consist of protein (N = 15 % ash free) and substances soluble in alcohol and ether.

Preliminary experiments with bean leaves showed that a similar separation could be readily effected. Immediately after weighing and counting, the batch of leaves was placed in a large beaker, covered with ether for one minute, and the ether drained off. The plasmolysed leaves[2] were then wrapped in a small piece of stout, well washed, filter cloth, and subjected to slowly increasing pressure in the Buchner press. The major part of the vacuole material, a clear amber-coloured fluid, was readily expressed. The filter cloth and contents were removed from the press, placed in a shallow enamelled iron tray, the cloth opened, and the exposed mass of flattened leaves allowed to absorb 0·002 *N* HCl (as with spinach, acid of this strength, in place of distilled water, was necessary to prevent the partial solution of the cytoplasmic proteins). The cloth was then again closed and pressure applied as before. This operation was repeated four times, so that the material was washed five times in all. All six extracts were combined, and the total volume measured.

The total time taken to extract each batch was 40 minutes; too short for post-mortem autolysis to have brought about any marked chemical change. How complete was the extraction is illustrated by Table IV (p. 392).

[1] In that paper the term "protoplasmic material" was meant to embrace the chloroplasts and other possible inclusions. "Cytoplasmic material" expresses this without ambiguity: it will, therefore, be used in future in place of the former term.

[2] The ether causes the cell protoplast to contract, the vacuole material passing freely through it. It is really false plasmolysis, since true plasmolysis, according to the definition of de Vries, implies the passage of water, and not dissolved vacuole material, through the protoplast.

Distribution of solids.

Vacuole material. An aliquot part of the vacuole extract was run into a tared beaker and evaporated to dryness on a water-bath. The beaker was then dried at 108° for 24 hours, cooled, and weighed.

Cell wall and cytoplasmic material. The leaf residue from the press was removed quantitatively from the filter cloth, and dried at 108° in an oven for 24 hours.

Distribution of protein N and non-protein N.

Protein N. As stated above, the residue from the press contains the cell wall and washed cytoplasmic material. The cell wall material contains no appreciable amount of N. The washed cytoplasmic material consists of protein together with substances soluble in alcohol and ether. Some of these latter, such as chlorophyll, phosphatides, etc. contain N, but the total amount, compared with the protein N present, is small, and not subject to diurnal variation. Any appreciable diurnal change of N in the pressed residue, then, can be ascribed to a change in the total amount of protein present.

The vacuole extract also contains protein. This was coagulated by boiling, filtered off and washed free from extract with water. The filter paper with the retained protein was then transferred to a Kjeldahl flask. Total protein N was taken as the sum of the N in the pressed residue and the coagulum obtained by boiling the extract.

Non-protein N. This was determined in the vacuole extract (after boiling to coagulate and remove protein) by Kjeldahl's method.

Table III. *Illustrating the separation of the protein N and non-protein N in a sample of* 149 *g. of leaves.*

Phaseolus vulgaris *var.* multiflorus.

		Percentage of total leaf N	N as a percentage of the fresh leaf weight
1st extract		4·64	0·0314
2nd "		1·35	0·0092
3rd "		0·89	0·0060
4th "		0·56	0·0038
5th "		0·33	0·0022
6th "		0·33	0·0022
Total non-protein N		8·10	0·0548
Total protein N	In coagulate	1·60	0·0106
	In residue	90·30	0·6084
Total leaf N		100·00	0·6738

Results.

The results of a series of three experiments, giving the diurnal variations in nine samples of leaves, are recorded in Tables IV–VIII. Table III is given to illustrate how complete is the separation of the protein N and non-protein N by the method described above. In this case each successive extract from the press was boiled to coagulate the protein, filtered and the N in it determined. It will

Table IV. *Showing details of samples used.*

Phaseolus vulgaris *var.* multiflorus.

Date picked	Number of leaves in sample	Weight 8 p.m. g.	Weight 6 a.m. g.	Change
14/15 Aug.	90	126·8	127·1	+0·3
„ „	96	133·6	130·4	−3·2
„ „	101	125·5	117·2	−8·3
20/21 „	93	110·1	108·8	−1·3
„ „	96	115·6	108·0	−7·6
„ „	115	116·1	116·3	+0·2
25/26 „	94	138·2	135·3	−2·9
„ „	91	111·5	114·2	+2·7
„ „	80	89·9	90·5	+0·6

Table V. *Variation of leaf total solids at night.*

Phaseolus vulgaris *var.* multiflorus.

(*Solids expressed as a percentage of the fresh leaf weight; change as a percentage of the* 8 *p.m. value.*)

Date picked	Cell wall + cytoplasmic material			Vacuole material			Total solids		
	8 p.m. %	6 a.m. %	Change %	8 p.m. %	6 a.m. %	Change %	8 p.m. %	6 a.m. %	Change %
14/15 Aug.	9·70	9·53	−1·7	4·41	4·45	0·0	14·10	14·00	−0·1
„ „	9·50	9·50	0·0	4·82	4·86	0·0	14·34	14·35	0·0
„ „	9·63	9·68	+0·5	4·41	4·70	+ 6	14·04	14·38	+0·2
20/21 „	9·80	9·75	−0·5	4·67	4·45	− 4	14·46	14·20	−0·2
„ „	10·31	9·96	−3·5	4·34	4·67	+ 6	14·67	14·63	0·0
„ „	10·35	9·85	−5·0	4·05	4·54	+11	14·40	14·40	0·0
25/26 „	10·60	10·27	−3·1	3·96	4·53	+13	14·56	14·79	+0·2
„ „	10·03	9·45	−5·8	3·67	4·09	+10	13·70	13·54	−0·1
„ „	10·63	10·17	−4·3	3·60	4·28	+17	14·23	14·44	+0·1
		Mean	−2·6 ±0·5		Mean	+ 6 ± 1·3		Mean	0·0

Table VI. *Variation of leaf total solids at night.*

Phaseolus vulgaris *var.* multiflorus.

(*Solids expressed as a weight per* 100 *leaves; change as a percentage of the* 8 *p.m. value.*)

Date picked	Cell wall + cytoplasmic material			Vacuole material			Total solids		
	8 p.m. g.	6 a.m. g.	Change %	8 p.m. g.	6 a.m. g.	Change %	8 p.m. g.	6 a.m. g.	Change %
14/15 Aug.	13·5	13·5	0·0	6·14	6·29	− 2	19·65	19·76	+0·1
„ „	13·2	12·9	− 2·3	6·70	6·60	− 1	19·90	19·50	−0·2
„ „	12·0	11·3	− 5·8	5·44	5·47	0	17·44	16·74	−0·4
20/21 „	11·6	11·4	− 1·8	5·55	5·20	− 5	17·13	16·61	−0·3
„ „	12·4	11·2	−10·0	5·23	5·26	0	17·67	16·46	−0·7
„ „	10·4	10·0	− 4·0	4·09	4·60	+12	14·54	14·55	0·0
25/26 „	15·57	14·78	− 5·3	5·84	6·52	+12	21·40	21·30	−0·0
„ „	12·28	11·86	− 3·3	4·50	5·13	+13	16·80	17·00	+0·2
„ „	11·95	11·50	− 4·1	4·05	4·84	+20	16·00	16·34	+0·2
		Mean	− 4·1 ± 0·7		Mean	+ 8·0 ± 2·0		Mean	0·0

Table VII. *Variation of leaf protein N, non-protein N and total N at night.*

Phaseolus vulgaris *var.* multiflorus.

(*Nitrogen expressed as a percentage of the fresh leaf weight; change as a percentage of the* 8 *p.m. value.*)

Date picked	Protein N 8 p.m. %	Protein N 6 a.m. %	Protein N Change %	Non-protein N 8 p.m. %	Non-protein N 6 a.m. %	Non-protein N Change %	Total N 8 p.m. %	Total N 6 a.m. %	Total N Change %
14/15 Aug.	0·576	0·562	−2·7	0·077	0·070	− 9	0·654	0·632	−3·4
,, ,,	0·550	0·546	−0·7	0·074	0·065	−12	0·623	0·611	−1·9
,, ,,	0·585	0·579	−1·1	0·076	0·071	− 7	0·661	0·650	−1·6
20/21 ,,	0·565	0·550	−2·7	0·066	0·059	−10	0·630	0·607	−3·7
,, ,,	0·608	0·592	−2·7	0·070	0·066	− 6	0·678	0·659	−2·8
,, ,,	0·589	0·575	−2·4	0·067	0·063	− 6	0·656	0·639	−2·6
25/26 ,,	0·623	0·614	−1·4	0·058	0·053	− 8	0·681	0·666	−2·2
,, ,,	0·595	0·584	−1·8	0·065	0·055	−15	0·660	0·639	−3·2
,, ,,	0·639	0·635	−0·6	0·067	0·061	− 9	0·706	0·696	−1·4
		Mean	−1·8 ±0·2		Mean	− 9·0 ± 0·6		Mean	−2·5 ±0·2

Table VIII. *Variation of leaf protein N, non-protein N and total N at night.*

Phaseolus vulgaris *var.* multiflorus.

(*Nitrogen expressed as a weight per* 100 *leaves; change as a percentage of the* 8 *p.m. value.*)

Date picked	Protein N 8 p.m. g.	Protein N 6 a.m. g.	Protein N Change %	Non-protein N 8 p.m. g.	Non-protein N 6 a.m. g.	Non-protein N Change %	Total N 8 p.m. g.	Total N 6 a.m. g.	Total N Change %
14/15 Aug.	0·803	0·793	−1·2	0·108	0·099	− 8	0·911	0·892	−2·1
,, ,,	0·765	0·742	−3·0	0·102	0·089	−13	0·892	0·831	−4·2
,, ,,	0·728	0·672	−7·6	0·094	0·082	−13	0·822	0·754	−8·3
20/21 ,,	0·669	0·643	−3·9	0·078	0·067	−14	0·747	0·711	−4·8
,, ,,	0·732	0·667	−7·6	0·085	0·074	−13	0·817	0·741	−9·3
,, ,,	0·595	0·582	−2·2	0·068	0·064	− 6	0·663	0·647	−2·5
25/26 ,,	0·918	0·884	−3·7	0·085	0·074	−13	1·001	0·958	−4·3
,, ,,	0·729	0·733	+0·6	0·079	0·069	−12	0·808	0·802	−0·7
,, ,,	0·718	0·719	+0·1	0·075	0·069	− 8	0·793	0·788	−0·6
		Mean	−3·3 ±0·6		Mean	−11·0 ± 0·7		Mean	−4·1 ±0·7

be seen that N in the last two extracts is only 0·0022 % of the fresh leaf weight, whereas the mean change in the protein N at night, as given by Table VII, is 0·010 % of the fresh leaf weight. Since in the experiments illustrated by Tables IV–VIII care was taken to standardise the conditions under which the non-protein N was extracted, it is justifiable to assume that the changes in protein N recorded are not due to differences in the degree of extraction of the water-soluble products from the leaf cells.

In Table IV two out of the nine diurnal samples taken show a difference of 6 % in weight, far greater than one could assign to translocation away from the leaf at night, and obviously due to different development of the basal and opposite leaflets. If comparison is made between Tables V and VI, and between VII and VIII it will be seen that Tables V and VII, based on method C are more reliable than Tables VI and VIII, based on method A, which confirms the conclusion given in the early part of this paper, that method A is unreliable unless sufficient samples are taken to allow probable errors to be calculated.

Discussion of Results.

Diurnal variations in the solids (Tables V and VI). Assuming that the cell wall material remains unchanged, it will be seen that there is a decrease in the cytoplasmic material at night, with a corresponding increase of soluble vacuole products. This is probably due to the breaking down of starch and protein. It is surprising that the total solids show no change at night. Since pods were forming at the time when these experiments were made there must have been some call on the leaves for carbohydrate. Assimilation in the weak light between sunrise and 6 a.m. ($1\frac{1}{2}$ hours) making up for losses at night, or the translocation of sugar taking place during the day, may possibly explain this.

Protein N and non-protein N. Table VII shows a fall in the protein N at night of 1·8 %, with a probable error of only 0·2 %. It can therefore be taken as an established fact that protein decomposition takes place in the leaf cells at night. Further, although it is not shown in the tables, the loss of protein is from the cytoplasm, and not from the small traces normally present in the vacuole. The non-protein N also shows a definite decrease of 9·0 % with a probable error of only 0·6 %. Both "ammonia N" and "amide N of asparagine" remained unchanged at 0·001 % and 0·005 % of the fresh leaf weight respectively, so that the products of the protein decomposition have undoubtedly been translocated to other parts of the plant. The establishment of these facts is of undoubted physiological importance, but further discussion is held over until the next paper, wherein the nature of these protein decomposition products is investigated.

Summary.

By properly controlled experiments it is shown that there is a definite fall n the protein content of bean leaves at night, due to the breaking down of the cytoplasmic material. The nitrogenous products of this protein decomposition are translocated away from the leaf.

REFERENCES.

Chibnall (1922). *Biochem. J.* **16**, 595.
—— (1923, 1). *J. Biol. Chem.* **55**, 333.
—— (1923, 2). *Ann. Bot.* **37**, 511.
Pigorini (1914). *Atti dei Lincei* (5), **23** (2), 433.
Schulze and Schütz (1909). *Land. Vers. Stat.* **71**, 299.
Suzuki (1897). *Bull. Coll. Agric. Tokyo*, **3**, 241.