XXIV. INVESTIGATIONS ON THE NITROGENOUS METABOLISM OF THE HIGHER PLANTS.

PART II.

THE DISTRIBUTION OF NITROGEN IN THE LEAVES OF THE RUNNER BEAN¹.

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IN the first part of this series [Chibnall and Schryver, 1921] a method of isolating part of the protein complex of fresh leaves was described. Subsequent work carried on with the same object, mentioned in that paper, in view, namely that of throwing light on the protein metabolism in the leaf, showed that it was necessary to investigate two problems in detail. Firstly the nature of the nitrogen retained in the residue of cellular matter after applying the above method remained to be determined, and secondly it was necessary to find out how far it is justifiable to compare leaves picked at different, and even unknown, periods of growth.

The present research on the leaves of the runner bean was primarily undertaken to solve these two problems. A method has been evolved whereby the whole of the nitrogenous material in the leaf is obtained in a state suitable for subsequent analysis. The water-soluble products are freed from all protein substances other than proteoses, about three-fourths of the protein N of the leaf is isolated as an amorphous powder, whilst the residue of the cell matter, containing 5-15 % of the total leaf N, is shown to contain only protein N. This makes it possible to estimate the total protein and water-soluble N in the leaf.

By applying this method at frequent intervals during the life history of the runner bean leaf, an attempt has been made to solve the second problem enumerated above. Not only has the ratio protein N to water-soluble N been found, but by suitable analysis either before or after hydrolysis, the distribution of the protein N in five groups, and of the water-soluble N in seven groups, has been determined.

A study of the protein groups indicates that the protein undergoes little, if any, change during the life history of the leaf, a fact that should be of assistance in future research into the chemistry of the leaf proteins. The water-soluble groups however are continually varying, indicating that comparisons of the composition of leaves, unless regard is paid to exact conditions

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of age and growth, is almost impossible [compare Jodidi, Kellogg and True, 1920].

As the research progressed it was found possible to extend the scope of enquiry to the diurnal variations, and the effects of starvation, the results of which throw considerable light on the protein metabolism in the leaf. The synthesis of protein in the leaf from nitrates appears to take place through the amino acids, whilst the products of protein degradation, in striking analogy to what is found in the animal kingdom, pass into what appear to be urea derivatives.

A certain amount of research has been performed in the past to throw light on most of the problems discussed in this paper [see Czapek, 1920], but it was nearly all carried out before the development of modern methods for estimating the nitrogenous bases and monoamino acids, so that comparison of results is of very little value.

I. GENERAL EXPERIMENTAL METHODS.

Materials used. The plant used was *Phaseolus vulgaris* v. *multifloris* (Scarlet Champion). The seeds were planted in unmanured ground following potatoes on the 28th April, 1921, at the Physic Gardens, Chelsea.

The seedlings appeared above the ground about 20 days later, and all ages of plants stated henceforth are calculated from the 18th May. The growth was normal, flowers appearing when the plant was five to six weeks old. At this time a few aphides appeared, but attention prevented them spreading, and all evidence of the disease had vanished three weeks later. By the middle of July it was found that although the inflorescence was normal, very few of the flowers had set, with the result that the number of pods formed was small, not averaging more than three or four per plant. This failure must be attributed to the abnormal dryness and high temperature of the atmosphere and not to the drought experienced during July, August and September, since the plants received abundance of water daily.

Methods of sampling. The procedure adopted was to pick the leaves from the plants selected, go lightly over the surface with a brush, remove all stems and take the total weight. It is from this weight, obtained before any appreciable evaporation from the surface of the leaves could have taken place, that the "N per fresh weight of the leaves," and hence all the figures given later in Tables XI and XIII, are calculated. The leaves were then counted, sorted out into six pans according to size, the content of each pan weighed, and the sample for grinding (200–250 g.) taken proportionately from each. By this means an average sample, giving a mean weight per leaf similar to that of the whole batch, was obtained. From several of the batches duplicates were taken to test the accuracy of the sampling and experimental methods used. The remainder of the batch was air-dried in an oven at 37° through which a slow stream of air was drawn.

Each batch picked was given a series number, which is retained throughout

in all subsequent operations. Details of the pickings, with the weather conditions, are given in Tables I and II. On account of the exceptionally fine weather conditions throughout the experiment the leaves were picked in a normal state without excess of moisture. Furthermore, the absence of high wind and storms kept the surface of the leaves clean and free from soil material. Only in one case, that of series 4, did rain fall in the preceding two or three days. In this rain fell during the night, but when the sample was taken in the morning no excess of moisture, as shown by lightly pressing between filter-papers, was found. Fresh weight determinations then can be considered as exceptionally good.

Dry weight and total nitrogen of the leaves. The air-dried remainder was used for this purpose. The dry weight was taken from a small sample placed for 24 hours in an oven heated to 108°.

The total N was determined by the modified Kjeldahl-Gunning method, whereby the nitrate N is first converted into amino-phenol by salicylic acid and sodium hyposulphite. The practical details followed were those given by Moore [1920].

General methods of manipulation (separation of protein, soluble products and cellular matter). Two methods which aim at this separation have so far been described, that of Osborne and Wakeman [1920] and the one communicated in the first paper on these investigations [Chibnall and Schryver, 1921]. In the former the leaves were passed through a Nixtamel mill three times; the pulp obtained mixed with water and returned twice more to the mill. The result was a green solution containing protein in colloidal suspension, from which the cellular matter was removed by centrifuging. The protein was afterwards flocculated by the addition of alcohol.

Briefly the second method consists of treating the minced leaves with ether-water and pressing out through thick muslin in a tincture press, whereby a green solution, containing most if not all of the water-soluble matter and part of the protein in colloidal suspension, was obtained. The protein was afterwards flocculated by warming. For the purpose of the present research this method, although easy to manipulate and rapid, was of little use unless the nitrogenous matter of the residue, about one-third of the total, could be separated from the cell matter for further investigation.

Osborne's method was certainly more thorough, the residue in this case containing only 18 % of the total N. It was inconvenient for the present purpose however, as it would have required the continuous use of a centrifuge. It was therefore decided to see if the ether-water method could not be improved.

Some cabbage leaves were accordingly treated by this method and the colloidal solution and residue examined microscopically, using a $\frac{1}{4}$ th objective.

The solution appeared colourless, with two distinct sets of particles. The first, deep green in colour and irregular in shape, were obviously fragments of disintegrated chloroplasts, and were large enough to be free from movement. The second were very numerous, showed no trace of green colour, and

were small enough to be in rapid Brownian movement. On warming to coagulate the protein the particles were all removed, and the coagulate appeared as a greyish granular mass in which the fragments of the green chloroplasts could be distinctly seen.

The residue to the naked eye showed large disintegrated lumps of leaf matter. One of the smaller of these was examined under the microscope. The outer layer of cells had their walls broken and the major part of the content expelled; those inside were unruptured but flattened. In addition the vacuole content of the latter had been largely expressed, even though the layer of protoplasm was unbroken.

It seemed clear from this examination that the rupturing of some of the cells was due to the action of the cutter in the mincing machine, and that the function of the ether-water was merely to kill the semi-permeable membrane of protoplasm in the remainder, so that the fluid content could be squeezed out by the press. The large quantity of N remaining in the residue therefore was due to the protoplasmic membrane remaining in the unruptured cells. Clearly then to reduce the N content of the residue it was necessary to increase the number of cells ruptured.

No access could be had to a mill of the type used by Osborne. The only one available was that known technically as an End-runner Mill, which is nothing more than a mechanical pestle and mortar. It was found that if 200 g. of cabbage leaves were broken up and fed slowly into this, then 100 cc. of water added, the whole was reduced to a very fine green pulp in about 20 minutes. On pressing through muslin a solution, deeper coloured than before, was obtained. The grinding and pressing was repeated on the residue, and the N in the two colloidal extracts and final residue determined. The first extract contained 74.2 %, the second 14.8 %, and the residue 11 % of the total N.

Under the microscope the solution appeared much as before, except that the fragments of green chloroplasts were much more numerous and irregular in size, ranging from quite coarse lumps, which appeared however free from cell matter, down to small, but distinctly green, particles in Brownian movement.

The residue appeared different. The large masses of leafy matter had disappeared. Lumps of cell matter however could still be seen under the microscope, though the number of unruptured cells had greatly diminished. In addition numerous shreds of broken cells, containing only infrequent portions of protoplasm, were to be seen.

It is on this property—that the cellular matter is not ground down to a powder (but only ruptured and torn so as to set free the cell content)—that the success of the method, which was used throughout the present research, depends. The thick muslin and the tincture press were found to be quite unnecessary. All that is required is a glass funnel opening to a diameter of about 8 inches and a piece of fine cotton lawn 18 inches square. The whole

of the liquid, with its heavy charge of colloidal matter, is expressed by very slight hand pressure in under a minute, leaving behind a pasty mass that can be easily removed from the lawn and returned to the mill for further grinding. The fact that this fine lawn can be used renders the method applicable for quantitative work, since none of the cell matter or protein can be retained in its pores, and the amount of water-soluble N absorbed is negligible.

Reference to Table IV will show how complete a separation was obtained in the present research. Six grindings of the samples (200–250 g.) were given and the colloidal solution, as a safeguard against the pores of the lawn being enlarged by the passage of the liquid under slight pressure, was run through a second piece spread out over another funnel.

In several of the intermediate batches ether-water was used in place of distilled water, but no benefit was experienced and it was later discarded. In case some of the cells should have escaped disruption, and thereby still be retaining their water-soluble contents, the residue from the sixth grinding was thrown into 200 cc. of boiling water. The cellular matter at once swelled up, imbibing the water, and on cooling this was squeezed through the same lawn as before. The operation was then repeated. In the earlier workings, when the total N in the residue was low, very little N passed into these two extracts. In the later however, when the residual N rose to 12 % and over, sufficient to make an appreciable difference was found in the first, though a negligible quantity was always found in the second. The protein was flocculated in the first six series by warming to 45°, but the temperature was afterwards raised to 60°, since it was found that the precipitate was then rendered almost granular, and could be readily filtered in a few minutes, whereas before it had taken about an hour. The filtering was accomplished by means of a Soxhlet extractor thimble standing upright in a glass funnel. After all the liquid had drained through, the retained protein was washed with two extracts from the boiled residue described above. After draining a second time the thimble was then transferred to an extractor described by Schryver [1908] and washed for three or four hours with 95 % alcohol. As first filtered off the protein is a pasty mass, part of which clings to the sides of the thimble, but the majority collects in a large button at the bottom. As the alcohol washes it the surface becomes dehydrated and shrinks, thereby coming away from the wall of the thimble. The whole can then be shaken out and broken, so as to allow the alcohol access to a large surface and thus hasten the washing. It was returned to the thimble and after a further three or four hours in this extractor the thimble was removed and stood overnight in absolute alcohol. In the morning it was placed in a Soxhlet apparatus and extracted with ether until all colouring matter was removed. The protein was then shaken out of the thimble, ground up in a small mortar, returned to the thimble and left 24 hours to dry. The weight of the thimble was determined before the operation, and the increase when it contained the air-dried protein was taken as the weight of the latter.

In the previous communication by Chibnall and Schryver [1921] it was found that after the colloidal protein of cabbage was flocculated by warming, a secondary flocculation could be obtained by raising the temperature to about the boiling point. The same was found to occur in the present case, but the amount was small, and diminished when the temperature of the first flocculation was raised to 60°. Experiment on a moderate quantity of this second flocculent substance prepared from the cabbage showed that it was formed from some water-soluble protein by coagulation, since it was insoluble in all the solvents tried, and the percentage of N in it was about 12. In the present case it was separated by filtering on a Buchner funnel, and the filter paper, with the retained coagulate, transferred to a Kjeldahl flask and the total N estimated. •

The aqueous extract, after boiling to remove all flocculent matter, was cooled, its volume taken, and stored in a stoppered bottle with a little chloroform and toluene for future use. As a precaution against any unknown reaction or separation on standing, the distribution of N in this was performed without delay. The alcohol and ether washings of the protein were evaporated down, transferred to a Kjeldahl flask and the total N estimated. The residue was air-dried at 37° in the same oven used for the main bulk of the leaves, as stated above. Total N was estimated by Kjeldahl's method.

It will not be out of place here to give some idea as to the time occupied in the separation. The grinding (10 mins. first, 6-7 mins. the others) and squeezing out take between 1 and 1½ hours; raising the temperature to 60°, 5 mins., filtering through the Soxhlet thimble 15 mins., boiling the filtrate 10 mins., and the separation of the coagulated protein about 15 mins. About two hours, therefore, after the leaf cells are killed the colloidal protein is in alcohol, the clear aqueous extract has been boiled, cooled and shaken up with antiseptics, whilst the residue, containing none of the water-soluble N, is drying at 37°.

DISTRIBUTION OF N IN THE WATER-SOLUBLE PRODUCTS.

The total N in solution. Determined by the modified Kjeldahl-Gunning method mentioned above.

Nitric nitrogen. Reduction by Devada alloy in alkaline solution in place of the more usual Schulze-Tiemann method was employed, as the same apparatus as that required for free ammonia and amide N could be utilised. This consisted essentially of a two-necked Claisen flask without side tube, connected by means of an ordinary Kjeldahl steam trap to a double-surface condenser leading into the distillate flask of 500 cc. capacity containing N/100 sulphuric acid. The apparatus was set up in duplicate, the two larger flasks being immersed in the same water-bath. Both were also connected to the same vacuum pump (Geryk), so that similar conditions as to *T* and *P* were maintained in each.

The procedure was as follows: 50 cc. of the extract was run into each

flask late in the afternoon, then 10 cc. of 30 % NaOH, and into *one only* a small quantity of powdered Devada alloy. In this latter the evolution of hydrogen proceeded steadily during the night, with reduction of the nitric N to ammonia. Sufficient quantity of *N*/100 acid had been run into the receiver flasks to cover the tube leading from the condenser and the apparatus closed. In the morning 400 cc. of ammonia-free distilled water was run in by means of a tap funnel, the water-bath warmed to about 45° and the duplicate apparatus evacuated. Distillation was allowed to proceed for about two hours, 200 to 300 cc. of liquid coming over. The difference between the ammonia found in the two receivers was taken as that due to reduction of the nitric N. How necessary is the blank distillation with soda will be seen from the fact that the ammonia coming over was about twice that obtained from the same volume of extract using magnesia (as below), indicating a very slight decomposition by the strong alkali of some nitrogenous bodies (proteoses?) in the solution. Duplicate readings of nitric N by this method, without any regard being paid to the exact *T* or *P*, agreed to 0.2 cc., an accuracy in the solutions containing the lowest concentration of nitric N of 1 : 100, sufficient for the present research.

Ammonia nitrogen. 50 cc. or, if sufficient solution was available, 100 cc. of the extract were run into the Claisen flasks, then 400 cc. of ammonia-free distilled water followed by sufficient cream of magnesia to make the solution distinctly alkaline. Duplicate determinations were made at the same time. The amount of ammonia measured was equivalent to 1.5–3.5 cc. of *N*/100 acid, a quantity sufficiently small to be affected by errors due to the action of the MgO on the proteoses (see Nash and Benedict [1921] for discussion on methods of estimating free NH_3 content of blood). The figures given in the tables therefore only indicate the very low concentration of ammonium salts.

Amide nitrogen (asparagine and glutamine N). Sachsse's [1873] method was employed, whereby the extract was boiled with 4 % HCl for two hours. Sufficient extract to contain one-fourth to one-third of the total N in solution was used for this purpose. After this mild hydrolysis the solution was concentrated *in vacuo* almost to dryness to remove HCl, transferred with about 400 cc. of ammonia-free water to the Claisen flask described above, then cream of magnesia added to make the solution distinctly alkaline. The total ammonia present was found by distillation *in vacuo*, and from this the free ammonia originally present in the solution, as estimated above, was deducted. When the details of this research were planned, it was not realised that any quantity of proteoses would be present. It is possible that these might give amide N under the conditions stated above [see Osborne and Nolan, 1920], but the fact that no amide N at all can be detected in the samples picked at night shows that the error under this heading is not appreciable.

Humin nitrogen and nitrogen precipitated by phosphotungstic acid. After the distillation for amide nitrogen the procedure follows that of Hausmann for the hydrolysis products of protein, as modified by Osborne and Harris [1903].

Monoamino nitrogen. The filtrate and washings from the phosphotungstic acid precipitation were made just alkaline to litmus with 30 % NaOH, then just acid by a few drops of glacial acetic acid. They were then concentrated *in vacuo* to about 100 cc. and made up to a standard volume. Monoamino N was determined by Van Slyke's method.

"*Other nitrogen.*" Calculated by difference; the value is therefore subject to the sum of the individual errors in the six determinations mentioned above.

Proteose nitrogen. Estimated by saturation with zinc sulphate in acid solution. The precipitate obtained was filtered off, washed with a saturated solution of zinc sulphate until the washings were colourless, and redissolved in water. The solution was then again acidified, saturated with zinc sulphate, the precipitate washed as before, re-dissolved in water and made up to a standard volume. Total N was then estimated in an aliquot part by Kjeldahl's method.

Nitrous nitrogen. This was tested for by both the Griess and meta-phenylenediamine reagents, but none was detected in any of the extracts (before or after heating) prepared in the present research.

DISTRIBUTION OF N IN PROTEINS.

Colloidal protein. Osborne and Harris' modification of Hausmann's method, referred to above, was used, monoamino N being determined by the method of Van Slyke.

Dried residue. When the total N in these (given later in Table IV) was determined they had not been washed in alcohol and ether to remove fats, lecithins, etc., since the extractors were being continuously used for work on the colloidal proteins. Series 7 B and 9 B, which were to be further investigated, were first washed with these solvents, when the N extracted was found to be respectively 0.60 % and 0.78 % of the total leaf N. These two extracted residues were then boiled up with 500 cc. of 1 % HCl for six hours under a reflux condenser, cooled, the undissolved portions filtered off, washed with water, and their N content determined by Kjeldahl's method. In terms of the total leaf N they contained 3.2 % and 4.13 % respectively. About three-fourths of the N left in the dried residue had therefore passed into solution. These extracts were then evaporated to a small bulk *in vacuo*, made up to 100 cc. so that they contained 20 % of HCl, hydrolysed for 16 hours, and the Hausmann numbers determined in a similar way to the colloidal protein above. They are given in Table VI later, and show that the N extracted was of protein origin. It is probable that the 3-4 % of N in the final residues mentioned above was also protein N that had escaped extraction by the dilute acid [compare Osborne and Wakeman, 1920].

Total protein and non-protein N. Previous work on the colloidal leaf proteins [Osborne and Wakeman, 1920; Osborne, Wakeman and Leavenworth, 1921; Chibnall and Schryver, 1921] indicates the possibility of a small

quantity of the N being of non-protein origin. In all the analyses therein mentioned, however, either hot or cold alkali was used for extraction, and since the resultant proteins have the properties of alkali-albumins, it is considered here that this "non-protein" N has been set free from the original protein complex by hydrolytic action. The constancy of the distribution of N in the colloidal proteins of the present research (Table V), especially in the starvation experiment to be described later, supports this view. In Table IV therefore the estimated total protein N is taken as the sum of the N in the colloidal precipitate, the dried residue and the protein coagulated by boiling.

There is evidence that more than one protein exists, amongst which may be cited the fact that the Hausmann numbers of the protein in the dried residues differ from those in the colloidal precipitate and also that a small part of the protein, as already mentioned, cannot be flocculated at 60°, but only precipitates when the liquid containing it is boiled. Furthermore, proteoses are present, but separate analysis to determine these could only be performed in the later series.

An analysis of the leaf N (series 9) in terms of protein N, proteose N and non-protein N is given in Table IX, whilst the distribution into seven groups after complete hydrolysis is given in Table X.

II. DESCRIPTION OF EXPERIMENTS.

Three main series of experiments have been carried out to determine:

- (a) Seasonal variations.
- (b) Diurnal variations.
- (c) Results of starvation.

(a) *The seasonal variations.*

These were studied by a complete analysis of eleven batches of leaves picked at frequent intervals during the life of the plant. Except for the change of temperature at which the colloidal protein was flocculated from 45° to 60°, as stated earlier, the conditions of the individual analyses remained standard throughout.

(b) *Diurnal variations (series 5-6 and 9-8).*

These were studied at the end of the seventh and eleventh weeks. The day reading was obtained by picking the batch about half-an-hour before sunset, when the leaves had been subjected to 14 hours' light; the night reading by picking half-an-hour before sunrise, when the leaves had been in the dark about six hours.

From a climatic point of view the conditions under which these series were taken were ideal. In each case no rain had fallen for several days previously, nor was there any dew during the night, the relative humidity at the time of the night pickings being 85 % and 75 % respectively. This being so, the large decrease in the dry weight of the leaves during the night (10 % in both cases) can be taken as due entirely to translocation away from the leaf.

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(c) *Starvation experiments (series 10).*

These were designed so to alter the conditions in the leaf that light would be thrown on the mechanism of either protein synthesis or degradation.

At the time that the batch for series 10 was picked two samples, 10 c and 10 d, were cut with long stems, placed in cups containing ordinary tap water so that their stems only were immersed, and left in a subdued light, 10 c for 100 hours and 10 d for 114 hours. In this way the leaves were kept quite fresh, but starved of nitrate and of sugar (other than that due to photosynthesis).

Table I. *Giving details of samples picked.*

Series number	Age above ground in days	Time of day when picked, G.M.T.	Date when picked	Condition of weather	Condition of plants
*1	14	8 A.M.	June 14	Sunny	Height 6-8 ins.
*2	21	"	" 21	Dull, no sun	Not very different from above
3	26	"	" 13	Sunny	Average height 12 ins. Just starting to climb
4	33	"	" 20	Dull, rainy night	Starting to flower
5	46	8.30 P.M.	July 3	Very fine day	Height 6-8 ft., flowers just developing
6	47	2.15 A.M.	" 4	Fine night	As above
7	60	3 P.M.	" 17	Very fine day	An appreciable increase of new shoots and leaves
†7N	66	2 A.M.	" 23	Fine night	Pods just starting to form
8	77	"	Aug. 3	" "	Fully grown. Fertilisation had not taken place on account of the heat. A few pods still forming
9	81	3 P.M.	" 7	Very fine day	Ditto
10	97	9 A.M.	" 23	Fine day	Only a few pods fully formed. Numerous new shoots and leaves still forming
11	143	"	Oct. 8	" "	Most of the leaves old and beginning to show signs of chlorophyll degeneration. A few new shoots and leaves

* Planted 14 days later than the rest of the plants.

† No analyses were made with this batch.

Table II. *Giving further details of samples picked.*

Series number	Number of plants used	Average number of leaves per plant	Average weight of leaves per plant in g.	Average weight of leaf in g.
1	68	5.7	5.3	0.935
2	50	7.6	5.4	0.715
3	25	15.3	15.6	1.027
4	13	30.5	29.7	0.975
5} day	12	56.3	50.9	0.904
6} night	12	53.6	55.6	1.086
7	12	109.0	90.0	0.827
7N	12	—	126.0	—
8} night	12	218.0	172.0	0.791
9} day	12	259.0	202.0	0.781
10	6	372.0	245.0	0.683

Table III. (Compare Fig. 1.) *Showing the dry weight and total nitrogen.*

Series number	Fresh weight of sample in g.	Percentage dry weight	Percentage of N per fresh weight	Percentage of N per dry weight
1	160	11.43	0.623	5.44
2	88	13.06	0.671	5.13
3	177	13.43	0.658	4.90
4	191	13.16	0.588	4.48
5} day	193	15.85	0.678	4.28
6} night	236	14.46	0.653	4.51
7	590	15.90	0.762	4.76
8} night	1365	14.42	0.656	4.53
9} day	1925	15.51	0.675	4.35
10	921	16.31	0.724	4.44
11	164	16.13	0.622	3.86

Table IV. *Distribution of protein and water-soluble nitrogen in the leaves.*
(In percentages of total leaf N.)

Series number	N of colloidal protein isolated	N of protein coagulated by boiling	N of residue	Estimated protein N *	N of alcohol and ether washings of colloidal protein	N remaining in solution after boiling
1	59.55	2.84	6.88	69.27	2.53	28.20
2	62.56	1.76	7.15	71.47	2.78	25.75
3	64.90	3.30	5.44	73.64	2.81	23.55
4	60.18	5.29	7.86	73.33	2.93	23.74
5A}	56.32	3.61	13.34	73.27	2.44	24.29
5B}	50.70	3.88	19.00	73.58	2.52	23.90
6A}	56.02	2.97	13.37	72.36	2.75	24.89
6B}	48.52	4.36	19.62	72.50	2.80	24.70
7A}	59.61	1.34	12.25	73.20	2.46	24.34
7B}	58.87	0.74	14.11	73.72	2.30	23.98
8A}	61.28	0.68	14.92	76.88	2.02	21.10
8B}	59.37	0.91	16.62	76.90	2.06	21.04
9A}	58.11	0.68	16.81	75.60	2.72	21.68
9B}	59.70	0.76	15.09	75.55	2.88	21.57
10A}	56.82	0.68	18.17	75.67	2.17	22.16
10B}	52.47	0.82	21.99	75.28	1.07	22.75
11	58.43	1.43	15.83	75.69	1.90	22.41
Starvation experiments:						
10c	49.55	1.79	12.30	63.64	2.22	34.14
10d	51.85	0.72	8.75	61.32	2.50	36.18

* The sum of the three columns on the immediate left.

Table V. *Percentage distribution of nitrogen in the colloidal protein.*

Series number	Amide N	Humin N	Basic N	Monoamino N	"Other" N
1	7.44	3.52	21.65	58.90	8.49
2	6.56	3.50	22.40	60.25	7.29
3	6.68	3.30	21.56	58.60	9.86
4	6.59	3.63	21.08	58.20	10.50
5A} day	6.68	4.18	20.55	59.67	8.78
6B} night	6.36	3.64	19.82	59.54	10.64
7B}	6.37	3.57	20.55	59.10	10.41
8B} night	6.14	3.55	19.33	59.25	11.73
9B} day	6.34	3.64	22.00	58.88	9.14
10B	6.50	3.82	21.78	59.22	8.68
*10c	6.60	3.56	21.63	58.50	9.71
11	6.40	3.82	21.32	58.50	9.96

* Starvation experiment.

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Table VI. *Showing the distribution of N in the products extracted from the residues by 1 % HCl (after complete hydrolysis).*

Series number	Amide N	Humin N	Basic N	Monoamino N	"Other" N
7B	8.38	5.73	25.18	48.20	12.53
9B	8.19	5.82	24.66	48.78	12.64

Table VII. *Distribution of the water-soluble nitrogen (in percentages of total leaf nitrogen). After hydrolysis with 4 % HCl for two hours.*

Series number	Ammonia N	Asparagine N	Nitric N	Humin N	Proteose + basic N	Mono-amino N	"Other" N
1	0.67	0.79	7.78	0.78	7.63	6.43	4.64
2	0.22	0.62	7.35	0.79	6.77	6.41	3.59
3	0.27	0.94	3.93	0.72	6.29	5.54	5.87
4	0.27	0.51	2.45	0.74	6.67	5.52	7.58
5A } day	0.47	0.49	2.71	1.32	6.81	9.57	2.92
5B }	0.36	0.43	2.96	0.66	6.25	9.25	3.99
6A } night	0.45	nil	3.89	1.19	6.60	10.37	2.39
6B }	0.34	nil	3.66	1.07	6.79	10.16	2.77
7A }	0.39	0.21	3.77	0.83	7.90	9.33	1.91
7B }	0.32	0.24	4.25	1.06	7.49	8.60	2.02
8A } night	0.34	trace	3.38	0.94	6.87	7.08	2.49
8B }	0.38	trace	3.74	1.07	6.62	7.05	2.18
9A } day	0.41	0.48	3.04	1.03	6.67	7.72	2.33
9B }	0.44	0.53	2.75	0.99	6.35	7.99	2.45
10B }	0.37	0.47	3.53	1.06	6.89	6.81	3.62
11	0.61	0.92	1.28	0.96	6.97	6.61	5.05

Table VIII. *Showing the percentage of nitrogen found in the colloidal precipitates.*

Series number	Percentage of nitrogen	Series number	Percentage of nitrogen	Series number	Percentage of nitrogen
1	11.42	6A }	11.84	9A }	11.66
2	11.44	6B }	10.75	9B }	11.71
3	11.76	7A }	11.75	10A }	11.54
4	9.73	7B }	11.81	10B }	11.36
5A }	10.88	8A }	11.84	10C } *	11.35
5B }	10.68	8B }	11.75	10D }	11.42

* Starvation experiment.

Table IX. *Showing the amounts of protein, proteose and non-protein nitrogen in the leaves of series 9 (age 14 weeks).*

	Percentage of total N
Colloidal protein	59.70
Protein in residue extracted by 1 % HCl	10.24
Undetermined in residue	4.13
Coagulated by boiling	0.76
Total protein N	74.83
Proteose N	4.35
Non-protein N, soluble in water...	17.22
Total	96.40
Soluble in alcohol and ether	3.60

Table X. *Showing the final analysis of the leaf nitrogen in series 9 B (in terms of total leaf N). After complete hydrolysis.*

	Ammonia N	Nitric N	Amide N	Humin N	Basic N	Mono- amino N	"Other" N
Colloidal protein	—	—	3.78	2.17	13.13	35.16	5.46
Residue	—	—	1.18	0.82	3.54	7.01	1.82
Proteoses, water- soluble N	0.44*	2.73*	1.26	1.08	2.78	9.01	4.25
Coagulate	—	—	—	—	—	Total N	0.76
Soluble in alcohol and ether	—	—	—	—	—		3.60
Total	0.44	2.73	6.22	4.07	19.45	51.18	15.89

* Determined before hydrolysis.

Table XI. (Compare Fig. 1.) *Showing the amounts of protein N, water-soluble N and the components of the water-soluble N in percentages of the fresh weight of the leaves.*

Series number	Protein N	Water- soluble N	Ammonia N	Nitric N	Aspara- gine N	Humin N	Protease + basic N	Mono- amino N	"Other" N
1	0.4315	0.1757	0.0042	0.0485	0.0049	0.0048	0.0475	0.0401	0.0289
2	0.4795	0.1728	0.0045	0.0493	0.0041	0.0053	0.0454	0.0430	0.1241
3	0.4846	0.1550	0.0017	0.0259	0.0062	0.0047	0.0414	0.0358	0.0386
4	0.4322	0.1396	0.0016	0.0144	0.0030	0.0043	0.0392	0.0325	0.0446
5A } day	0.4967	0.1647	0.0027	0.0192	0.0031	0.0067	0.0444	0.0637	0.0237
5B }	0.4989	0.1620							
6A }	0.4725	0.1626							
6B } night	0.4732	0.1613	0.0026	0.0247	0.0	0.0072	0.0438	0.0670	0.0168
7A }	0.5575	0.1855							
7B }	0.5620	0.1828							
8A } day	0.5045	0.1382	0.0024	0.0234	0.0	0.0066	0.0443	0.0463	0.0152
8B }	0.5045	0.1380							
9A }	0.5102	0.1463							
9B } day	0.5100	0.1458	0.0028	0.0196	0.0034	0.0067	0.0439	0.0523	0.0172
10A }	0.5477	0.1605							
10B }	0.5450	0.1647							
11	0.4708	0.1394	0.0038	0.0080	0.0058	0.0059	0.0423	0.0411	0.0314

The last seven columns are the mean of the respective A and B values

Table XII. *Showing variations introduced in the distribution of the water-soluble N of series 10 by starvation.*

(Figures given are in percentages of the total leaf N)

Series number	Hydrolysis		Nitric N	Ammonia N	Aspara- gine N	Amide N other than asparagine N	Humin N	Protease N	Basic N	Mono- amino N	"Other" N
	Strength of HCl	Time in hours									
10A & B	4%	2	3.53	0.37	0.47	—	1.06	4.59	2.30	6.81	3.62
10c (1)	4	2	3.42	0.46	0.49	—	0.88	(3.60) ¹	0.67	6.95	17.66
10c (2)	20	16	3.42	0.46	0.49	5.69	1.71	—	5.03	8.88	8.46
10d (1)	—	—	—	—	—	—	—	3.52	—	—	—
10d (2)	20	16	(3.42) ²	(0.46) ²	(0.49) ²	6.57	1.72	—	5.61	9.64	8.27
9B (1) ³	4	2	2.73	0.44	0.53	—	0.99	4.35	2.00	7.76	2.77
9B (2) ³	20	16	2.73	0.44	0.53	0.73	1.08	—	2.78	9.01	4.25

¹ Estimated by comparison with 10d (1).² Not determined; assumed, by comparison with 10c, to be unchanged.³ Extract 9B was used as none of 10A or 10B was available.

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Table XHI. *Showing the diurnal variation in the N of series 5-6 and 9-8, in percentages of the fresh weight of the leaves.*

Series number		Total N	Protein N	Water-soluble N	Ammonia N	Nitric N	Asparagine N	Humin N	Proteose + basic N	Mono-amino N	"Other" N
5A } 5B }	day. Mean	0.6780	0.4980	0.1634	0.0027	0.0192	0.0031	0.0068	0.0444	0.0637	0.0237
6A } 6B }	night. Mean	0.6530	0.4730	0.1620	0.0026	0.0247	0.0	0.0072	0.0438	0.0670	0.0168
Diurnal difference		-0.0250	-0.0250	-0.0014	-0.0001	+0.0055	-0.0031	+0.0004	-0.0006	+0.0033	-0.0069
9A } 9B }	day. Mean	0.6750	0.5101	0.1460	0.0028	0.0196	0.0034	0.0067	0.0439	0.0523	0.0172
8A } 8B }	night. Mean	0.6560	0.5045	0.1392	0.0024	0.0234	0.0	0.0066	0.0443	0.0463	0.0152
Diurnal difference		-0.0190	-0.0056	-0.0068	-0.0004	+0.0038	-0.0034	-0.0001	+0.0004	-0.0060	-0.0020

+ Indicates that there is a diurnal increase. - Indicates that there is a diurnal decrease.

Table XIV. *Showing the difference between certain duplicate readings in Tables IV and VII. (Expressed as a percentage of the mean value of the two readings.)*

Series number	Protein N	Water-soluble N	Nitric N	Monoamino N	"Other" N
5A } 5B }	0.44	1.69	8.44	3.37	25.0
6A } 6B }	0.15	0.83	5.91	2.05	14.0
7A } 7B }	0.75	1.48	11.30	8.49	5.5
8A } 8B }	0.0	0.15	9.60	0.43	12.0
9A } 9B }	0.0	0.50	9.54	3.50	6.0
10A } 10B }	0.50	0.77	—	—	—

The results of the above experiments are given in Tables III to XI. All the figures in the hydrolyses are the means of duplicates. Since the fresh weight of the sample taken for grinding might be too low on account of evaporation from the surface of the leaves whilst sorting, the distribution of N was calculated in terms of the total N. To convert this distribution into one in terms of the fresh weight of the leaves, as is given in Tables XI and XIII, the appropriate value for "N per fresh weight" given in Table III was used. In connection with these tables the following definitions must be noted:

(1) "*Amide N*" in Tables V and VI. This is used in the standard way to denote the ammonia set free on the complete hydrolysis of proteins, etc.

(2) "*Asparagine N.*" Ammonia obtained by hydrolysis with 4 % HCl for two hours (after deduction of free ammonia nitrogen). (See reservation *re* Osborne and Nolan given on p. 350.)

(3) "*Amide N other than asparagine N.*" In Table XII this is the ammonia set free on complete hydrolysis minus that estimated as asparagine in (2), and as free ammonia.

(4) "*Monoamino N*" refers only to the amino N that is estimated by Van Slyke's method.

(5) "*Other N*" is the N not estimated in any of the other groups given. Calculated by difference.

(6) "*Protein N*" is the sum of the N in the colloidal precipitate, dried residue and coagulated protein found by boiling the extract (see p. 351).

Fig. 1 illustrates Table XI. The curves are drawn to show a value for about 9 a.m. to 11 a.m. (after 6-8 hours daylight). Whenever only sunrise and sundown values have been determined, the value shown on the curves (Fig. 1) is the mean of the two.

*Sampling and experimental errors in the distribution of N*¹. In no case was it possible to take more than duplicate samples of the same batch of leaves, so that probable errors, based on Peter's formula, cannot be obtained. Table XIV however shows the differences between duplicate samples of the more important groups given in Tables IV and VII calculated as a percentage of the mean reading. The protein N shows a maximum difference of 0.75 % only, so that all the changes indicated in Fig. 1 can be taken as significant. The water-soluble N shows a maximum difference of 1.69 %. The nitric N shows a maximum difference of 11.3 % and a minimum of 5.91 %. This is much greater than the experimental error, and must be due to variation of the concentration in different leaves. As none of the changes indicated by Fig. 1 is less than 20 % they can be regarded as significant.

Monoamino N shows a maximum difference of 8.49 %, although that in the other four readings does not exceed 3.5 %. The experimental error here is undoubtedly high, due to the small volume of N actually measured, but a variation of 12 % or over should be fairly significant.

"Other N" is calculated by difference, and shows a maximum of 25 %. Considering that it is subject to all the errors in the rest of the N groups, a variation of 50 % is required to be at all significant.

III. DISCUSSION OF RESULTS.

The main results, as indicated in the tables and figure, may be summarised as follows:

(a) *Seasonal variations.* (Tables I, II, III, V; Fig. 1.)

(1) Total N and protein N, calculated as percentages of fresh weight, vary with growth, decreasing when this is very rapid or pods are forming.

(2) The distribution of N in the colloidal protein remains practically unchanged throughout the series.

(3) Asparagine N and free ammonia N remain low throughout the series.

(4) Nitric N and monoamino N vary directly with the protein N, indicating that they may be connected with protein synthesis. Similarly the "other N" varies inversely as the protein N, indicating that it may be connected with protein degradation.

¹ Sampling errors for dry weight and total N were not obtained in the present research. Earlier work on the leaves of the broad bean showed, for a batch of 250 g., the following probable errors calculated by Peter's formula: dry weight, 0.48 %; N per dry weight, 0.63 %; N per fresh weight, 0.91 %.

(5) The water-soluble products show considerable variation throughout the series, indicating that comparison of leaves from plants of different age is impossible [see Jodidi and colleagues, 1920].

(b) *Diurnal variations.* (Table XIII.)

- (1) There is a diminution of total solids at night.
- (2) This is accompanied by a diminution of total N and protein N.
- (3) There is a distinct rise in the nitric N at night.
- (4) The asparagine N disappears at night.
- (5) The other water-soluble products remain more or less unchanged in amount during the night.

(6) The most significant diminution of nitrogenous products at night must be assigned to the proteins, indicating translocation of unchanged protein or its decomposition products.

The results for series 9-8 are not so reliable, since three days elapsed between the pickings. The conclusions to be drawn from them are similar to those stated above, except that there is a loss of both water-soluble N and monoamino N. This difference may be connected with the fact that pods were forming at the time.

(c) *Starvation experiments* (series 10). Tables IV, V and XII.

(1) There is a considerable decrease of protein N, with increase of water-soluble N.

(2) The distribution of N in the colloidal protein remains unaltered.

(3) Nitric N and ammonia N remain unaltered.

After mild hydrolysis:

(4) Asparagine N and monoamino N remain unchanged.

(5) Proteose N and basic N have decreased.

(6) There is a large increase of "other N," equivalent to the decrease in the protein N.

After complete hydrolysis:

(7) The "other N" is greatly reduced, and allowing for proteoses in solution, half of the loss reappears as ammonia (amide N other than asparagine N in Table XII) and one-third as bases.

Perhaps the most interesting of these results is that the protein disappears, and is replaced, not as one might expect by amide N and amino N, which remain more or less constant, but by N products of undetermined composition, given under the heading "other N." Light is thrown on the nature of these products by the different results of mild and complete hydrolysis. It would appear that they consist, in large part, of substances that fulfil the following conditions:

After mild hydrolysis:

- (1) Yield no free ammonia, *i.e.* contain no acid-amide linkage $R-CO-NH_2$.
- (2) Give no precipitate with phosphotungstic acid.
- (3) Give no increase of free amino groups capable of reacting with nitrous acid.

After complete hydrolysis:

(4) Break down giving about one-half their N as ammonia and one-third as bases.

This great increase of free ammonia on complete hydrolysis suggests a urea derivative in which the amino groups are coupled to form compounds capable of resisting mild hydrolysis. The ureides are substances of this type; further they are, as a class, strongly acidic and capable of forming salts. As an example take barbituric acid,

This substance contains both replaceable imino and methylene hydrogen atoms, and only on prolonged hydrolysis breaks down giving ammonia from the urea nucleus. A condensation product of such an acid with one or more of the nitrogenous bases might possibly resist disruption on mild hydrolysis, and so give no precipitate with phosphotungstic acid due to liberated bases. In the absence of further chemical evidence this view can only be regarded as a speculation, but it is worth noting that these products appear to be present in small quantities in the unstarved leaf extract. A complete hydrolysis (see 9 B (1) and (2), Table XII) of this extract gives an increase of ammonia equivalent to 17 % of the proteose N, a value three times greater than one would expect from published data concerning the amide N content of proteoses. Furthermore, Fosse [1912, 1913, 1914] has demonstrated the presence of traces of urea in the leaves of higher plants.

Summarising the results under (a), (b) and (c) above, it would appear that the nitrogenous metabolism of the leaf runs on the following lines:

This will explain the following points brought out in the above discussions:

(1) The graphs of the nitric and monoamino N in Fig. 1 vary with that of the protein N.

(2) In spite of rapid protein degradation at night, there is very little change in most of the constituents of the water-soluble N.

(3) There is an accumulation of "other N" during the fourth and fifth weeks when protein degradation appears to be rapid, also later in the season when the plant is becoming aged, and translocation less active.

(4) In the starvation experiment, though protein degradation is rapid, there is no increase in amide N or monoamino N, and, since translocation away from the leaf has stopped, there is a large increase in "other N."

Protein metabolism, however, does not depend on N alone, and due regard must be paid to the supply of carbohydrate and other substances when interpreting some of the results outlined above. Thus in the starvation experiments,

since nitric N is present, it might be asserted that protein synthesis would go on until this was used up. But in this case the leaves were starved of sugar, and since they were placed in a subdued light photosynthesis would be weak. In all probability then, protein synthesis had stopped for lack of carbohydrate [compare Suzuki, 1898]. Similarly the accumulation of nitric N at night is probably due to the fact that starch, stored during the day, has been partially or entirely used up, so that the supply of carbohydrate for protein synthesis is reduced or is stopped.

The low concentration of free ammonia and asparagine N observed throughout the series confirms the work of Emmerling [1887] on the broad bean, and of Kosutany [1897] on the vine. The researches of Wasilieff [1901] and Prianischnikoff [1899] have shown that the growth of seedlings depends in part on asparagine derived from the decomposition of the reserve proteins in the cotyledons. This may indicate that the function of asparagine in the general metabolism of the plant is connected with growth, so that the total disappearance at night, an observation confirmed by Kosutany, may be due to translocation away from the leaf to the growing points or roots. The starvation experiments, where the asparagine N remains unchanged, support this view, and show that this substance is not derived from the decomposition of the leaf proteins. No indication as to its origin can be deduced from the results of the present research, but they lend support to the theory of Butkewitsch [1909] that its formation is to remove excess of free ammonia.

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