LII. INVESTIGATIONS ON THE NITROGENOUS METABOLISM OF THE HIGHER PLANTS. PART VI.

THE RÔLE OF ASPARAGINE IN THE METABOLISM OF THE MATURE PLANT.

BY ALBERT CHARLES CHIBNALL.

From the Biochemical Department, Imperial College of Science and Technology, London, and the Connecticut Agricultural Experiment Station, New Haven.

(Received February 21st, 1924.)

INTRODUCTION.

OUR knowledge of the nitrogenous metabolism of the higher plants is still in a very incomplete state. Such as we have is based more or less entirely on experiments made with germinating seedlings. Obviously the metabolic changes thus shown are only of limited application and cannot be applied to the mature plant, since the nitrogenous metabolism of seedlings concerns mainly the decomposition of already existing protein stored up in the cotyledons and its regeneration at the growing points. As soon as this reserve is exhausted, as it usually is after the 14th–20th day, the plant metabolism develops so as to include the synthesis of protein and other organic nitrogenous materials from the simple substances, such as nitrates, which the root-system can extract from the soil. The focus of metabolic activity is then transferred to the leaves, which become the seat of protein, as well as of carbohydrate, synthesis. For an adequate knowledge of the metabolic processes occurring in the mature plant then, it is of the first importance that light should be thrown on the nitrogenous metabolism in the leaf.

The function of the protein in the leaf, and the nature of the compounds with which the leaf supplies the other parts of the plant to make up for losses due to living processes and for growth has received but small attention in the past.

Borodin [1878], using microchemical methods, stated that the disappearance of protein in leaves was accompanied by the appearance of asparagine. Schulze [1880], who cut young shoots from the birch and placed them with their cut ends in water for 10–12 days, found an accumulation of asparagine in the leaflets. Later Schulze and Bosshard [1885] repeated the experiment with young oat plants, and found an increase of asparagine at the expense of protein (see Table I).

Table I. *Showing the protein decomposition in young oat plants kept in a dark room for eight days. (Schulze and Bosshard.)*

	In percentages of the dry weight	
	Fresh plants, taken from field and analysed direct	Plants cut and left with stems in water in a dark room for 8 days
Total N	4.12	4.50
Protein N (Stutzer) *	3.51	1.46
Asparagine N (Sachse)	0.15	1.69
Other N (diff.)	0.46	1.35

Butkewitsch [1908], enlarging on this early work of Schulze's, showed that the increase of asparagine corresponded roughly to the decrease of protein and pointed out that this was analogous to the decomposition of the cotyledon reserve proteins in the germinating seedling.

In the above-quoted experiments the root-system had been detached, but even so the normal translocation of substances from the leaves to other parts of the plant and *vice versa* was still possible. Therefore, the accumulation of asparagine cannot be correlated definitely with the metabolism of the leaf proteins.

Only one experiment so far has been made with leaves completely detached from the plant, so that any accumulation of substances can be ascribed definitely to metabolic processes in the leaf itself. Suzuki [1897] showed that during the night there was a decrease in the leaf protein [for criticism of this statement see the preceding paper, Chibnall, 1924], and, since the amounts of asparagine and what he referred to as "amido" compounds, did not increase, he concluded that the protein had been resolved into these substances, which were then translocated away from the leaf. Negative results of this nature, however, unsupported by further experimental data, cannot be considered as sufficient evidence to support such a far-reaching conclusion.

In the preceding paper [Chibnall, 1924] the author has shown, by properly controlled experiments, that there is undoubtedly a fall in the protein content of runner bean leaves at night; also that the products of this decomposition are not stored up in the leaf, but translocated. In this present paper experiments have been devised so as to throw light on the chemical nature of these leaf protein decomposition products. It is found that about one-half of the leaf protein nitrogen has passed into asparagine, and about one-quarter into other amino bodies of undetermined composition. Asparagine, then, is one of the chief products of the nitrogen metabolism of the mature leaf. The conclusion is reached that this substance is, in the mature plant, one, and probably the most important, of the means whereby nitrogen in a form suitable for easy resynthesis of protein is conveyed from one part of the mature plant to another. This has already been shown to be the case in germinating seedlings.

GENERAL STATEMENT OF THE PROBLEM TO BE INVESTIGATED.

In the present paper the author offers no evidence concerning protein synthesis in leaves, but is concerned only with the composition and fate of its decomposition products.

The problem was attacked in the following way. There is evidence, collected and confirmed in the preceding paper [Chibnall, 1924], that the total amount of protein present in the leaf undergoes a decided diminution during the night, and that, whatever the nature of its decomposition products, these are not stored up in the leaf but translocated, since the water-soluble nitrogen in the leaf shows very little diurnal change.

The object of the present research was to devise means whereby these decomposition products could be submitted to chemical analysis.

The method adopted was to cut from the plant leaves with long petioles and allow them to remain with their petioles in water for four or five days. The leaves remained fresh and turgid throughout, so that respiration, though probably not normal, was not interfered with [compare Spoehr and McGee, 1923]. Although the conditions thus produced are artificial they have the advantage that translocation to and away from the leaf is eliminated. Therefore the products of nitrogenous metabolism, which normally appear to migrate, accumulate, and so become available for chemical analysis.

GENERAL EXPERIMENTAL METHODS.

Materials used. The plant used was the runner bean (Scarlet Champion), *Phaseolus vulgaris* var. *multiflorus*. The seeds were planted about the end of April, 1922, in unmanured ground at the Physic Garden, Chelsea, following plants of the same species the previous year. Growth was normal, and during August and early September, when the samples of leaves used during the present research were picked, the plants were in process of pod formation. The one experiment made during 1923 was with leaves from plants used in the research described in the preceding paper [Chibnall, 1924]. Flower buds were just about to form at the time of picking.

Description of experiments. Large batches of mature leaves with long petioles were cut from the plant. The ends of the petioles were then at once cut under water, and bunches of about 30 leaves were placed in glass jugs filled with tap water so that the petioles were immersed. The leaves themselves were well above the water and both their upper and lower surfaces were freely exposed to the air, so that respiration was not interfered with.

They remained thus for four or five days in either strong diffused daylight or in the dark, depending on the conditions of the experiment being performed. The tap water in the jugs was changed daily, and about $\frac{1}{4}$ " from the end of each petiole was also removed daily, so as to be sure that bacterial contamination did not interfere with the free supply of water through the petiole to the leaf.

At the end of the prescribed time the leaves were withdrawn from the jugs, care being taken not to wet the leaf surfaces, the petioles removed, and the leaves subjected to the analysis described below.

At the same time that the leaves for the above experiments were removed from the plant, control batches of leaves were analysed directly after cutting, so that the change in the nitrogenous products of the leaves placed with their petioles in water could be followed. Details of samples used are given in Table II.

Table II. *Giving details of the samples of bean leaves used.*

Phaseolus vulgaris var. multiflorus.

Date picked (10-12 a.m.)	Number of days with petioles in water	Total weight of leaves in sample (g.)
5 August, 1922	4 in light	1200
8 " "	0	2010
12 " "	4 in dark	2400
23 " "	0	5240
25 " "	5 in light	2740
26 " "	5 in dark	1870
7 Sept. "	4 in light	2020
11 " "	0	400
12 July, 1923	0	258
12 " "	4 in dim light	132

Separation of water-soluble products from the leaves. The fresh leaves, free from all extraneous moisture, were first weighed, and then a sample of about 200 g. removed for determinations of dry weight and total nitrogen.

The water-soluble products were then extracted from the remainder by the earlier ether-water method of Chibnall and Schryver [1921]. The extract from the press was boiled to coagulate the proteins, and subsequently filtered. The residue from the press was then extracted three times successively with boiling water, the mixture, after cooling, being pressed out in the tincture press. By this means the major part of the water-soluble products are obtained in aqueous solution free from all protein and colloidal material. The object of the present research was qualitative rather than quantitative, and in no case is it certain that the water-soluble products were quantitatively removed. The amount remaining in the pressed residues was, however, very small.

The above practical details refer to samples of leaves picked in 1922. In the one experiment made in 1923 the newer method of extraction, described in the previous paper [Chibnall, 1924], which gives a quantitative result, was used.

Total protein and non-protein nitrogen. From previous work on the leaves of the runner bean [Chibnall, 1922] it is clear that the water-soluble nitrogen, as separated above, is non-protein nitrogen (proteoses may be present). The nitrogen of the pressed residues and of the coagulum obtained by boiling the extract, is mostly protein nitrogen, for small amounts due to chlorophyll, etc. can be neglected in this present research.

Distribution of nitrogen in the water-soluble products. Total N, ammonia N, and "amide N of asparagine" were determined by the methods described in a former paper [Chibnall, 1922]. After removal of the ammonia set free from the asparagine by the 4 % HCl hydrolysis (according to Sachsse's method), the excess of magnesia was dissolved by adding a small excess of glacial acetic acid. This solution was then made up to a standard volume, and free amino N determined by the method of Van Slyke. Urea was determined by the urease method of Folin and Wu, but it was present only in minute amounts, and did not increase in the leaves placed with their petioles in water.

Results are given in Tables III and IV.

Isolation of asparagine. It is to be remembered that Sachsse's method gives only the nitrogen in the acid amide linkage CO-NH_2 and is not necessarily evidence of the presence of asparagine. Actual evidence that asparagine was present was obtained by the crystallisation method of Schulze and Winterstein [1910, 1, 2]. The plant extract was treated with neutral lead acetate in slight excess and allowed to stand 24 hours. The resulting precipitate was then centrifuged off and washed. The residual liquid and washings were next treated with mercuric nitrate until no further precipitation occurred, the solution being kept nearly neutral to litmus by means of sodium hydroxide. After standing 24 hours the precipitate was centrifuged off, washed and decomposed with H_2S . The precipitate of HgS was centrifuged off, and the clear liquid made neutral by a few drops of strong ammonia. It was then evaporated on the water-bath to a small bulk, a drop or two of ammonia being added occasionally to keep the reaction neutral. The concentrated solution was then allowed to crystallise slowly in a vacuum desiccator. 500 cc. of the extract made 7th September were first treated in this way. From preliminary analyses this solution contained 2.28 g. of N and 0.294 g. of "amide N" (Sachsse). The distribution of "amide N" (Sachsse) was followed in the separation, and 64.2 % of it was carried down in the mercury precipitate. The yield of recrystallised asparagine ($\text{C}_4\text{H}_8\text{O}_3\text{N}_2 \cdot \text{H}_2\text{O}$) was 0.75 g. or 30 % of that given by Sachsse's method. It gave the following analysis.

	Found	Calculated for $\text{C}_4\text{H}_8\text{O}_3\text{N}_2 \cdot \text{H}_2\text{O}$
Amide N (Sachsse)	9.01 %	9.34 %
Amino N (Van Slyke)	9.51	9.34

Since portions of the various solutions had been withdrawn for analysis this yield was not a maximum. Therefore a further isolation was made from 660 cc. of the extract made 12th August containing 3.20 g. of total N and 0.457 g. of "amide N" (Sachsse). The preparation was recrystallised twice from hot water. The final product, after drying for some days in a vacuum desiccator, weighed 1.958 g. It gave the following analysis:

	Found	Calculated for $\text{C}_4\text{H}_8\text{O}_3\text{N}_2 \cdot \text{H}_2\text{O}$
Water of crystallisation (by heating to 108° for $1\frac{1}{2}$ hours)	12.12 %	12.00 %
Total N (Kjeldahl) ...	18.44	18.68
Amide N (Sachsse) ...	9.16	9.34
Amino N (Van Slyke) ...	9.48	9.34

The asparagine remaining in the mother liquids from the second and third recrystallisations was converted into the Cu salt by boiling with $\text{Cu}(\text{OH})_2$, filtering and allowing to cool. The copper salt gave on analysis 19.59 % of Cu; calculated for asparagine 19.50 %. The gross yield of pure asparagine ($\text{C}_4\text{H}_8\text{O}_3\text{N}_2 \cdot \text{H}_2\text{O}$) was 2.394 g. or 48.8 % of that indicated by Sachsse's method. No trace of glutamine was found. As the previous isolation from the extract made 7th September had shown that only 60–70 % of the "amide N" (Sachsse) was precipitated by the mercuric nitrate the yield stated above can be regarded as satisfactory for that fraction. An attempt was also made to isolate asparagine from one of the control plant extracts. 1375 cc. of the extract made 23rd August containing 3.20 g. of total N and 0.176 g. of "amide N" (Sachsse) were submitted to the above treatment, but no crystals of asparagine were obtained.

The application of this method for the isolation of asparagine and glutamine from plants has been chiefly employed by Schulze and his co-workers at Zürich. With germinating seedlings of lupins, in which asparagine makes up nearly two-thirds of the total water-soluble products, Schulze [1880] obtained a yield corresponding very closely with that given by Sachsse's method. When, however, he used the green parts of mature plants his yield of crystalline asparagine was greatly reduced. Thus Schulze and Bosshard [1885], from 1 kilo. of fresh oat plants, were unable to isolate any asparagine, though Sachsse's method indicated 0.27 g. of "amide N." When similar plants had been left with cut stems placed in water for eight days, thereby showing a tenfold increase of "amide N" by Sachsse, they obtained only a 20 % yield of crystalline asparagine. Butkewitsch [1908], who repeated this work of Schulze's, was more successful, and obtained from the plants left with their cut stems in water yields of crystalline asparagine equal to 40–45 % of that shown by Sachsse, a result similar to the present author's quoted above.

As to the nature of the remaining 30–40 % of "amide N" (Sachsse) in the bean extract of 12th August described above, there is, of course, no direct evidence. That part of it at least is derived from asparagine is probable, since it is hardly to be supposed that a complete separation of one single substance can be carried out by the method outlined above from such a complex mixture as the water-extract of a leaf.

As far as is known, no other substances that have been isolated from plants are capable of yielding ammonia when submitted to such mild hydrolysis. Urea, and, as a general class, hydantoins and the alloxan series are stable under this treatment. But it must not be forgotten that, as will be shown later, asparagine is undoubtedly the most important and most abundant product of protein metabolism in the leaf; consequently simple polypeptides containing this amino-acid may also be present. Thus Fischer has prepared several dipeptides containing asparagine, whilst Thierfelder and von Cramm [1919] have recently synthesised several polypeptides of glycine, alanine and glutamine. Such bodies have not yet been isolated from the plant, but the possibility

Table III. *Showing the increases in the various forms of non-protein N in the leaves placed with their petioles in water.*

Phaseolus vulgaris var. multiflorus.

(In percentages of total leaf N.)

Date picked	No. of days with petioles in water	Total non-protein N	Ammonia N	Amide N (Sachse)	Free-amino N
8 August, 1922	0	13.54	0.28	0.64	6.00
5 "	4 in light	28.43	0.47	4.42	14.18
	Increase	14.89	0.19	3.78	8.18
8 "	0	13.54	0.28	0.64	6.00
12 "	4 in dark	31.70	0.45	4.53	14.07
	Increase	18.16	0.17	3.89	8.07
23 "	0	16.50	0.30	0.89	6.81
25 "	5 in light	29.84	0.37	4.46	15.77
	Increase	13.34	0.07	3.57	8.96
23 "	0	16.50	0.34	0.89	6.81
26 "	5 in dark	35.14	0.57	5.85	15.82
	Increase	18.64	0.23	4.96	9.01
11 Sept. 1922	0	16.75	0.46	1.06	7.40
7 "	4 in light	31.60	0.65	4.08	14.27
	Increase	14.85	0.19	3.02	6.87
12 July, 1923	0	14.05	0.30	0.75	—
12 "	4 in light	37.74	1.01	5.63	—
	Increase	23.69	0.71	4.88	—

Table IV. *Showing the increases in the various forms of non-protein N in the leaves placed with their petioles in water.*

Phaseolus vulgaris var. multiflorus.

(In percentages of total non-protein N.)

Date picked	No. of days with petioles in water	Total non-protein N	Ammonia N	Amide N (Sachse)	Free-amino N
8 August, 1922	0	13.54	2.00	4.72	44.30
5 "	4 in light	28.43	1.65	15.55	49.85
	Increase	14.89	- 0.35	10.83	5.55
8 "	0	13.54	2.00	4.72	44.30
12 "	4 in dark	31.70	1.42	14.29	44.37
	Increase	18.16	- 0.58	9.57	—
23 "	0	16.50	2.00	5.98	41.30
25 "	5 in light	29.84	1.24	14.60	50.27
	Increase	13.34	- 0.76	8.62	8.97
23 "	0	16.50	2.00	5.98	41.30
26 "	5 in dark	35.14	1.62	16.65	45.00
	Increase	18.64	- 0.38	10.67	3.70
11 Sept. 1922	0	16.78	2.74	6.32	44.10
7 "	4 in light	31.60	2.06	12.87	45.16
	Increase	14.82	- 0.68	6.55	1.06
12 July, 1923	0	14.05	2.16	5.30	—
12 "	4 in light	37.74	2.79	13.85	—
	Increase	23.69	0.63	8.55	—

of their accounting for part of the "amide N" (Sachsse) must not be overlooked.

Until further experimental evidence is available then, only 60-70 % of the "amide N" (Sachsse) obtained in the present bean extracts can be safely ascribed to asparagine.

DISCUSSION OF RESULTS.

The results given in Tables III and IV can be summarised as follows:

(1) In the case of the leaves placed with their petioles in water there is, in terms of total leaf N, a large increase in non-protein N, with a corresponding decrease in the protein N.

(2) This increase is accounted for in large part by asparagine and free amino nitrogen.

(3) In terms of total water-soluble N the only increase is in the "amide N" (Sachsse); the concentration of the free amino N remaining more or less unchanged.

It is clear that one of the main products of the protein oxidation is ammonia. Free ammonia, however, is toxic to plants, so that it is probably removed by the formation of asparagine, which, even in high concentration, is innocuous to plants.

It must be remembered, however, that in the case of the leaves placed with their petioles in water conditions are artificial. As Pfeffer rightly emphasises [1897, p. 443] a substance that normally remains intact may be drawn into metabolism when the plant is starved, or the external conditions are altered in some way. Consequently the protein decomposition observed in the leaves that have had their petioles placed in water may be due not to the normal course of nitrogenous metabolism in the leaf but to abnormal conditions, chief among them being carbohydrate deficiency. That a continuous production of CO_2 is necessary in such leaves, even when kept in the dark, follows from some experiments of Spöehr and McGee [1923], who showed that although respiration decreases, it still remains active. Therefore, the possibility of the decomposition of protein and the production of CO_2 therefrom must not be overlooked; such a phenomenon is well known in animal metabolism.

Two facts, however, seem to indicate that this disappearance of protein is due, at any rate in part, to the normal course of protein metabolism in the leaf. In the first place the increase of total protein observed in leaves during the day, followed by a decrease at night [see Chibnall, 1924], indicates that protein decomposition in the leaf goes on continuously, and is only masked during the day by the fact that the rate of protein synthesis exceeds that of protein degradation. In the second place there is, in the case of the leaves kept with their petioles in water, a continuous and more or less equivalent fall both in the protein of those kept during the daytime in strongly diffused daylight, and in the protein of those kept continuously in the dark. As protein

synthesis in both these cases must be negligible, owing to the low concentration of nitrates and ammonium salts in the leaves when picked from the plants, it may be inferred that light has had no direct influence on the protein degradation. The disappearance of protein in these leaves, then, may not be due entirely to "starvation," but in part at least, due to the normal metabolism of the living cells. Table III, and the statements given earlier under general experimental methods, show that at least 35–40 % of the N from the protein undergoing degradation changes has passed into asparagine. Therefore it seems just to conclude that in the normal mature leaf there is a continuous production of asparagine from the reserve proteins.

Repeated researches since the time of Boussingault have shown that the concentration of asparagine in the green parts of plants grown under normal conditions is extremely low. Only under abnormal conditions, which produce a deficiency of carbohydrate, such as etiolating the plants, does any accumulation of asparagine occur [see Pfeffer, 1897, p. 462; Butkewitsch, 1908]. Since this is so it would appear that the asparagine formed by the protein degradation must be continuously translocated away from the leaf, and used by other parts of the plant for the protein synthesis necessary for growth and maintenance of life.

This fact is of great interest when compared with our already existing knowledge of the nitrogenous metabolism in germinating seeds, and in the formation of pods and seeds. In a series of papers Schulze and his co-workers at Zürich have shown that the metabolism of the reserve proteins in the seeds leads to a great increase of asparagine (or in certain exceptional cases glutamine), which is then translocated away to the growing parts and, as they believed, resynthesised to protein and other complex nitrogenous substances necessary for the newly formed cells. Similarly, during the ripening of seeds asparagine is translocated to the pericarp, and is then used for synthesis of protein in the seeds [Schulze and Winterstein, 1910, 2; Schulze, 1911].

All this evidence indicates that the rôle of asparagine in the metabolism of the mature plant is that of a translocatory substance, and that it is the chief medium whereby nitrogen in a form suitable for subsequent metabolic processes can be transferred from one part of the plant to another. Pfeffer considered that this was its chief function in the metabolism of seedlings. There is ample evidence, however, that asparagine can play another important part in plant metabolism. As Prianischnikoff [1916] has already shown with young plants, should conditions, such as derangement of the normal protein metabolism in the plant, give rise to ammonia, this substance, which is toxic in excess, is removed as asparagine. Table IV shows that the same reaction can occur under abnormal conditions in the mature leaf. As Prianischnikoff points out, in this respect asparagine in plant metabolism corresponds to urea in animal metabolism. The analogy, however, is misleading since urea cannot be utilised again by the animal body but is excreted, whereas asparagine remains stored up in the plant, and, under favourable conditions, can be subsequently utilised.

SUMMARY.

The decomposition products arising from protein metabolism in bean leaves have been investigated. These consist in large part of asparagine and other substances of undetermined composition containing free amino nitrogen.

The rôle of asparagine in the metabolism of the mature plant is discussed. It appears to be the chief agent whereby nitrogen in a form suitable for resynthesis of protein is conveyed from one part of the plant to another.

REFERENCES.

- Borodin (1878). *Bot. Zeitung*, **36**, 802.
Butkewitsch (1908). *Biochem. Z.* **12**, 314.
Chibnall (1922). *Biochem. J.* **16**, 344.
—— (1924). *Biochem. J.* **18**, 387.
Chibnall and Schryver (1921). *Biochem. J.* **15**, 60.
Pfeffer (1897). *The Physiology of Plants*, Vol. I; translated by Ewart.
Príanischnikoff (1916). *Moscow Agric. Instit.* **10**; *Abst. Chem. Soc.* 1917, **1**, 616; *Ann. Rep. Chem. Soc.* 1917, 217.
Schulze, E. (1880). *Land. Jahrb.* **9**, 689.
—— (1911). *Z. physiol. Chem.* **71**, 31.
Schulze, E. and Bosshard (1885). *Z. physiol. Chem.* **9**, 420.
Schulze, E. and Winterstein (1910, 1). *Abderhalden's Handbuch der biochemischen Arbeitsmethoden*, **11**, 513.
—— (1910, 2). *Z. physiol. Chem.* **65**, 431.
Spoehr and McGee (1923). *Studies in Plant Respiration and Photosynthesis. Carnegie Inst. Publications*, 325.
Suzuki (1897). *Bull. Coll. Agric. Tokyo*, **3**, 241.
Thierfelder and von Cramm (1919). *Z. physiol. Chem.* **105**, 58