CCXXXV. THE NITROGEN METABOLISM OF THE LUPIN SEEDLING.

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THE following investigation was undertaken for two reasons; to follow the nitrogen metabolism of the young plant through successive stages of growth and thus to arrive at some estimate of the part played by different nitrogenous metabolites, and to provide a preliminary survey for the much more complicated problem—at what stage and by what means is the pyrrole ring synthesised in the plant?

The first stages of germination of a seed containing reserve protein involve the breakdown of that protein. It becomes a matter of considerable interest therefore to examine the various products of this breakdown and to gain some knowledge of the nitrogen compounds which are produced by the plant from its reserves, prior to their use in synthesis. It is apparent, as will be shown, that protein synthesis begins after about the 18th day of germination, when the seed reserves have been (for the lupin) largely depleted. The main seat of metabolic activity of such synthesis is probably in the leaves. In a young seedling, in which the leaves form a small proportion of the bulk of the plant, it is misleading to confine analysis solely to these parts, and up to the 82nd day of growth the whole seedling has been taken for analysis. The material from each sowing (about 12 plants) was mixed, discarding any obvious abnormalities. In this way minor differences, inseparable from the growth of individual plants, were eliminated. In the lupin, asparagine represents about one-half of the protein decomposition products in the seed, and the nitrogen metabolism of the lupin seedling is therefore peculiar in this respect, but asparagine is probably only one of several labile intermediate substances of protein breakdown, and the subsequent course of protein re-synthesis may give some qualitative idea of this process in other seedlings.

EXPERIMENTAL.

Lupin seeds (*Lupin luteus*), were grown in large pots containing a granular soil and sand mixture in a cool greenhouse throughout the summer and early autumn of 1930. Twelve pots, each producing ultimately about a dozen plants, were used for each sowing. Regulation of the temperature of the greenhouse ensured approximately the same conditions throughout the experiment. In

one lot only were conditions adverse; Lot 1, a 31 day period lasting from October 25 to November 29, which coincided with an exceptionally cold spell of weather. Artificial heat above the average cool temperature was not favourable, and growth was stunted accordingly.

Preparation and analysis of material.

The plants were released from the soil by turning out the pot and gently disengaging the root and root-hairs as much as possible without breaking. Ten minutes' immersion in water and very gentle cleaning with the fingers were sufficient to free the seedlings entirely from soil. They were quickly air-dried on sheets of blotting-paper, and measured for total length, number of leaves and lateral roots.

After 1 minute's immersion in boiling water to arrest growth and enzyme action, the seedlings were dried as completely as possible on blotting-paper and transferred to spirit. This enables analyses to be undertaken at leisure, and avoids the disadvantage of high temperature drying of the partially wet material; it also affords a means of isolating the alcohol-insoluble asparagine which appears in quantity after a few days' growth. A deposit of asparagine appears at the bottom of the jar, and in the subsequent stages—rapid air-drying at 50°—the rest of the asparagine crystallises out on the dry material, and can readily be detached. The asparagine-free material is ground to powder in an end-runner mill and dried at 100° to constant weight. Analyses for total nitrogen (Kjeldahl) only were carried out on the dry material.

Extraction of material. The dried and weighed material was intimately ground in an end-runner mill with 100 cc. of ammonia-free water for 20 minutes and filtered through fine muslin. The process was repeated twice more, the last residue being pressed as dry as possible.

Residue. This was dried at 100° to constant weight and analysed for total nitrogen.

Filtrate. The combined filtrates were made up to 500 cc. with ammonia-free water, made antiseptic with a little toluene and analysed.

Total nitrogen. Of the dry material weights averaging 0.02 g., or of the extract 10 cc. evaporated to dryness in the Kjeldahl flask, were taken. The modified Kjeldahl method used by Robinson [1929], was found satisfactory. A clear liquid was always obtained after 20 minutes' boiling, and boiling was continued for a further 10 minutes. Distillations were carried out in the usual apparatus with the addition of 30 cc. of ammonia-free water, 5 cc. of 25 % sodium sulphide, 5 cc. of *N* copper sulphate, and 25 cc. of alcohol, aerated by acid-washed air during the 20 minutes' boiling, the ammonia being received in *N*/10 H_2SO_4 . Controls had established the accuracy of this procedure.

Protein. 10 cc. of extract were diluted to 100 cc. with absolute alcohol, added slowly with constant shaking. After standing for 36 hours the precipitated protein was collected in a Jena glass crucible and dried in a desiccator to constant weight. Nitrogen was calculated in this protein as 12.8 %.

Proteose. The method of Wasteneys and Borsook [1917] was found most suitable. 20 cc. of extract were employed.

Nitrate. This was determined on 50 cc. of extract by reduction with Devarda's alloy as recommended by Chibnall [1922].

Amino-acids. The Sørensen method of estimation of amino-acid content was finally adopted, 10 cc. being used for each titration.

Ammonia. Free ammonia, which is usually small in amount, is best estimated by the Folin aeration method, using solid anhydrous sodium carbonate. 30 cc. of extract *plus* 2 g. of anhydrous sodium carbonate were aerated in the Folin apparatus for 1 hour at 15°, the ammonia being received in 25 cc. of $N/100 \text{ H}_2\text{SO}_4$.

Amides. The amide content was taken as represented by that amount of ammonia *minus* the free ammonia, which is yielded by the Folin method after 5 hours' boiling with 4 % HCl , by the method of Sachsse [1873]. 5 cc. of extract and 20 cc. of acid were taken for each estimation.

Asparagine. A considerable proportion of the asparagine, when present, crystallises out from the material in spirit, and, after the removal of the seedlings, can be filtered off through a Jena glass crucible, dried and weighed. The remainder crystallises on the surface of the seedlings during the air-drying and can be detached with a small brush, collected and weighed.

Fig. 1.

Examination of the curves, Fig. 1, shows that over a period of 81 days' growth from the seed, protein, insoluble nitrogen compounds, and asparagine represent by far the greater part of the total nitrogen.

In the seed, soluble protein and nitrogenous material insoluble after the three extractions with water account for the whole of the nitrogen.

After 5 days' growth, soluble protein has decreased sharply, and this is the more marked at 8 days, when there has been a fall of protein nitrogen from 59.2 % (in the seed) to 20.2 %. After a slight fluctuation, the level falls still further, until at the 18th day it is only 9.8 %, the minimum value (Table I).

Table I. *Variation in protein content.*

	Age (days)	Protein dry weight g.	Protein as % of total solid	Protein- nitrogen as % of total solid	Protein- nitrogen as % of total nitrogen
X.	0	13.4	29.6	3.8	59.2
B.	5	—	—	—	—
A.	8	0.395	12.95	1.65	20.02
F.	13	0.420	16.21	2.07	23.4
C.	18	0.410	14.68	1.88	9.81
G.	22	0.740	35.34	4.52	27.8
E.	25	0.320	13.69	1.75	12.01
D.	31	0.735	18.5	2.24	15.03
H.	82	0.665	22.37	2.34	18.5

(N.B. Protein nitrogen is calculated as 12.8 %.)

Following closely this decrease in protein there is a sharp decrease in insoluble nitrogenous material, which reaches its minimum on the 22nd day at 14.9 % (original seed value = 41.09 %) (Table II).

Table II. *Variation in content of insoluble nitrogen compounds.*

Age (days)	Nitrogen as % of dry residue	Residue-nitrogen as % of total dry weight	Residue-nitrogen as % of total nitrogen
0	8.8	2.64	41.09
5	—	4.94	43.96
8	8.8	3.98	61.3
13	7.07	3.61	41.5
18	6.7	3.97	20.7
22	5.3	2.42	14.9
25	8.54	6.34	43.55
31	8.22	5.8	38.9
82	5.29	3.33	26.3

Concomitantly with this fall asparagine increases rapidly during the early days of growth. It is absent from the ungerminated seed, but after 18 days' growth the asparagine represents 53.7 % of the total nitrogen. This is its maximum value, and during subsequent growth it diminishes rapidly in the next 12 days, corresponding to a synthesis of protein, and thereafter diminishes slowly. Asparagine appears, therefore, to represent the chief metabolic product in the first stages of growth and to represent, with the protein, the greater part of the nitrogenous material of the seedling (Table III).

Table III. *Variation in asparagine content.*

	Age (days)	Total solid dry wt. g.	Asparagine g.	Asparagine- nitrogen g.	Asparagine- N as % of dry wt.	Asparagine- N as % of total N
X.	0	—	0	0	0	0
B.	5	—	—	—	—	—
A.	8	3.0487	0.2521	0.0535	1.75	21.23
F.	13	2.5924	0.2377	0.0503	1.94	22.3
C.	18	2.7928	1.3292	0.2818	10.29	53.7
G.	22	2.0945	0.4724	0.1002	4.78	29.4
E.	25	2.3367	0.3146	0.0667	2.99	20.5
D.	31	3.9735	0.4087	0.0867	2.18	14.6
H.	82	2.9729	0.189	0.04	1.35	10.6

Proteoses are seen to be an immediate decomposition product of the protein and insoluble nitrogenous material, the curve for proteose nitrogen rising from zero in the seed to a maximum at 14.2 % after 18 days' growth. While protein is being rapidly synthesised during the next 7 days, proteoses diminish, but in the subsequent slower growth of the young plant, proteoses increase gradually with a corresponding increase of protein, keeping as would be expected, at a lower level throughout (Table IV).

Table IV. *Variation in proteose content.*

Age (days)	Total nitrogen of extract g.	Nitrogen other than protein or proteose g.	Nitrogen of protein plus proteose g.	Nitrogen of protein g.	Nitrogen of proteose g.	Proteose- nitrogen as % of total solid	Proteose- nitrogen as % of total nitrogen
5	0.198	0.127	0.071	0.050	0.021	0.07	0.85
8	0.175	0.084	0.091	0.053	0.038	0.15	1.72
13	0.253	0.125	0.128	0.052	0.076	2.72	14.2
18	0.241	0.112	0.129	0.095	0.034	1.68	10.3
22	0.273	0.220	0.059	0.046	0.013	0.55	3.78
25	—	—	—	—	—	—	—
31	0.335	0.218	0.117	0.069	0.048	1.66	13.13
82	—	—	—	—	—	—	—

The behaviour of the labile metabolic products shows an interesting parallel. Always low and never representing in total more than 9 % of the total nitrogen, and averaging ammonia and amino-acids, 0.2 %, amides and nitrates 3 %, they appear in the seedling after 5 days' growth, and are at their maximum during the period of rapid change, 8 to 18 days, corresponding to the very rapid fall of protein and the equally rapid rise of asparagine. With the exception of nitrates they maintain a fairly constant low level throughout the subsequent slower periods of growth. Nitrates tend to increase slightly as the seedling acquires more leaves and leaf metabolism thereby increases (Tables V, VI, VII and VIII).

The apparently insignificant part played in the seedling by amides, amino-acids, nitrates and ammonia, as suggested by the curves, is probably however misleading, for at no time is it to be expected that these substances, as much the elements of synthesis as of degradation, would accumulate. Thus, while the breakdown of protein to amino-acids, amides and ammonia may be rapid

Table V. *Variation in amide content.*

	Age (days)	Total solid dry wt. g.	N as amide plus ammonia g.	N as ammonia g.	N as amide g.	Amide-N as % of total solid	Amide-N as % of total nitrogen
X.	0	—	0	0	0	0	0
A.	8	3.0487	0.0105	0.0026	0.0079	0.259	3.15
F.	13	2.5924	0.0084	0.0021	0.0063	0.243	2.76
C.	18	2.7928	0.0091	0.0014	0.0077	0.276	1.41
G.	22	2.0945	0.0063	0.0014	0.0049	0.234	1.44
E.	25	2.3367	0.007	0.0013	0.0057	0.244	1.65
D.	31	3.9735	0.0091	0.0012	0.0079	0.199	1.34
H.	82	2.9729	0.0056	0.0014	0.0042	0.141	1.11

Table VI. *Variation in ammonia content.*

	Age (days)	Total solid dry wt. g.	Ammonia g.	Ammonia nitrogen g.	Ammonia-N as % of total solid	Ammonia-N as % of total nitrogen
X.	0	—	0	0	0	0
A.	8	3.0487	—	0.0026	0.084	1.02
B.	5	2.3628	—	0.00059	0.0257	0.26
F.	13	2.5924	—	0.0021	0.081	0.93
C.	18	2.7928	—	0.0014	0.050	0.22
G.	22	2.0945	—	0.0014	0.067	0.41
E.	25	2.3367	—	0.0013	0.055	0.38
D.	31	3.9735	—	0.0011	0.029	0.19
H.	82	2.9729	—	0.0014	0.047	0.73

Table VII. *Variation in nitrate content.*

	Age (days)	Total solid dry wt. g.	Nitrate- nitrogen g.	Nitrate- nitrogen as % of dry wt.	Nitrate- nitrogen as % of total nitrogen
X.	0	—	0	0	0
B.	5	—	—	—	—
A.	8	—	—	—	—
F.	13	2.5924	0.0071	0.2746	3.15
C.	18	2.7928	0.0156	0.56	2.92
G.	22	2.0945	0.0171	0.8155	5.01
E.	25	2.3367	0.0071	0.3044	2.09
D.	31	3.9735	0.0199	0.5013	3.36
H.	82	2.9729	0.0227	0.7656	6.01

Table VIII. *Variation in amino content.*

	Age (days)	Total solid dry wt. g.	Amino- nitrogen g.	Amino- nitrogen as % of dry wt.	Amino- nitrogen as % of total nitrogen
X.	0	0	0	0	0
B.	5	—	—	—	—
A.	8	3.0487	0.0023	0.076	0.92
F.	13	2.5924	0.0022	0.083	0.954
C.	18	2.7928	0.0016	0.056	0.29
G.	22	2.0945	0.0008	0.040	0.24
E.	25	2.3367	0.0010	0.044	0.30
D.	31	3.9735	0.00103	0.026	0.17
H.	82	2.9729	0.0005	0.016	0.13

and extensive, the removal of these compounds in an actively growing plant would be equally rapid. Thus the curve representing their presence at any one stage would be expected to show a low and fairly constant percentage.

Table IX. *Distribution of nitrogen as percentage of total nitrogen.*

	Age (days)	Total nitrogen %	N as NH ₃ %	N as nitrate	N as amide	N as pro- tein	N as pro- teose	N as amino- com- pounds	N as aspa- ragine	Insol. N	Total
X.	0	6.42	0	0	0	59.2	0	0	0	41.1	100.3
B.	5	9.89	0.26	—	—	—	—	—	—	43.9	—
A.	8	8.24	1.02	—	3.15	20.2	0.85	0.92	21.2	—	—
F.	13	8.7	0.93	3.1	2.76	23.4	1.72	0.95	22.3	41.5	96.6
C.	18	19.16	0.22	2.9	1.41	9.81	14.2	0.29	53.7	20.7	103.2
G.	22	16.29	0.41	5.03	1.41	27.8	10.3	0.25	29.4	24.9	99.4
E.	25	14.56	0.38	2.06	1.65	12.0	3.7	0.30	20.5	43.5	84.2
D.	31	14.49	0.19	3.36	1.34	15.0	—	0.17	14.6	38.9	—
H.	82	12.64	0.37	6.01	1.11	18.5	13.1	0.13	10.6	26.3	—

The rôle of asparagine in the metabolism of the leguminous seed has been investigated by several workers. Schulze [1880, 1911; Schulze and Winterstein, 1910] have noticed its formation in considerable quantity concomitantly with the breakdown of protein on germination. They suggest that its removal corresponds to a re-synthesis of protein, but this has not, so far, received quantitative treatment. Chibnall [1924], in his examination of the mature leaf of the runner bean, finds the same phenomenon and suggests that asparagine is a translocation product whereby nitrogen is conveyed from regions of protein catabolism to those of re-synthesis. The close relationship, shown by the curves, Fig. 1, between the fall of asparagine and proteose and protein re-synthesis in the lupin seedling is striking confirmation of this.

The seed, during the rapid degradation of reserve protein, concentrates its labile nitrogen in the form of the soluble, and hence mobile, compound asparagine. When the peak of catabolic activity is passed, and the young plant has energy free for the building up of its own material, asparagine is quickly removed by further chemical change, and synthesis of proteose and protein begins, passing presumably through the stages of amide, nitrate and ammonium salt.

SUMMARY.

1. Insoluble nitrogenous material, protein and asparagine, represent the bulk of the total nitrogen of the lupin seedling in the seed and in the early stages of growth.

2. A close relationship between decrease of insoluble and protein nitrogen and increase of asparagine is observed.

3. Protein synthesis begins after 16 days' growth and is accompanied by a fall in asparagine content and a rise in proteose content.

4. Ammonia, amides, amino-acids and nitrates are absent from the ungerminated seed. They appear immediately growth begins, rise to a low maximum in a few days and then maintain a constant lower level.

REFERENCES.

- Chibnall (1922). *Biochem. J.* **16**, 344.
—— (1924). *Biochem. J.* **18**, 395.
Robinson (1929). *Biochem. J.* **23**, 1099.
Sachsse (1873). *J. prakt. Chem.* **6**, 118.
Schulze (1880). *Landes. Jahrb.* **9**, 689.
—— (1911). *Z. physiol. Chem.* **71**, 31.
—— and Winterstein (1910). *Z. physiol. Chem.* **65**, 431.
Wasteneys and Borsook (1917). *J. Biol. Chem.* **29**, 25.