THE FORMATION OF ASPARAGINE AND ITS PHYSIOLOGICAL SIGNIFICANCE IN THE LUPINE SEEDLING

KUAN-JEN YANG[1]

Introduction

Making studies of the nitrogen metabolism, the plant physiologists have obtained an interesting result that asparagine can be accumulated to a considerable extent from shoots of growing seedlings germinating in the dark for 2 to 3 weeks. This result has been the subject of much discussion in physiology for a number of years.

Different theories have been proposed to explain the physiological role of asparagine and the chemical process involved in its accumulation in plant metabolism, but the procedure and the mechanism of accumulation and other substances related in plant metabolism are not yet understood even at the present time.

According to Vickery (1945), asparagine, the amide of amino succinic acid, had been found in asparagus over one hundred years ago and was named so thereafter. Mothes (1926) credited success to Hartig as having extracted this substance from seedlings and isolated it for the first time in crystalline form. Mothes thought that asparagine was a translocation product of nitrogen in young plant tissues.

After extensive microchemical studies on the presence and absence of asparagine in all parts of a large number of plants, Pfeffer (1872) attempted to express some views by concluding that: (1) Protein degradation in plants, because of the formation of asparagine, differs from urea in animals. (2) Asparagine is the primary, not the end product of protein break-down and should also be considered as a storage form of nitrogen. (3) Judged from its presence and solubility, asparagine is the main translocation product of nitrogen substance. It moves to growing regions where it combines with carbonhydrates (glucose) to form protein (Cited by Chinall, 1924).

Schulze (1919) found that asparagine can make up to 25% the dry weight of etiolated lupine seedlings. Following the suggestion of Bordin, he supposed

(1) Assistant research fellow, Institute of Botany, Academia Sinica.

that the accumulation of asparagine in the etiolated seedlings is a response to starvation conditions under which protein is hydrolyzed and the resulting amino acids are used as substrates for respiration. He also found that the amount of asparagine accumulated greatly exceeds the amount already contained in the reserve protein of seeds and that the release of asparagine by protein hydrolysis must take place at the expense of other amino acids.

Similar reaults were obtained by Prianischnikov (1922). His view is that asparagine is developed by the seedlings in response to the presence of ammonia in the plant and that asparagine synthesis is in fact a detoxification mechanism.

Consequently, the behavior of the formation of asparagine in plant is interpretable in terms of Prianischnikov's hypothesis that asparagine is synthesis in plant in response to an increase in ammonia, and provides a mechanism whereas the ammonia concentration is ordinarily maintained below a toxic level.

Prianischovikov (1922) also found that by supplying ammonium salts, an increased amount of asparagine was obtained. He suggested that asparagine might result from the union of ammonia and aspartic acid with loss of water but that aspartic acid might not be the particular substance on which ammonia would act.

Although the effects of various factors upon the formation of asparagine in seedlings have been investigated by several investigators, yet nobody seems to pay much attention to the relationship between asparagine and amino acids through the method of paper chromatography.

The purpose of the present study is to gather fundamental information from the asparagine formation and its physiological significance in the seedlings of *Lupinus luteus*. There is a considerable body of literature on the nitrogen metabolism of different plants, but so far as the writer is aware, such study of lupine previously undertaken seems next to none. Yet lupine seeds are rich in high quantity of proteins and can produce, in their seedlings, a kind of amide; namely, asparagine. Owing to this reason, they are available as samples for study under this subject.

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Materials and Methods

1. *Germination of the Seeds*

The lupine seeds (*Lupinus luteus*, yellow lupine) used in this study were supplied by the Taiwan Agriculture Research Institute.

In preparation for germination, the lupine seeds were sorted out by hand. Broken fragments as well as seeds with broken coating were discarded. The selected seeds were soaked for 20 minutes in a dilute uspulum solution (0.1%) to prevent mold growth. Then the seeds were washed four times with distilled water at 20°C to 21°C. They were then soaked for 6 hours in distilled water.

Seeds that had been sterilized and soaked were germinating in the sterilized petri dishes containing moistened filter paper. When the seedlings had reached to 2 cm. in height, they were transferred to the culture vessels.

Forty seedlings were placed on a double layer of cheesecloth in a beaker (500 ml.) covered tightly on the top. The beakers were filled with distilled water or culture solution. The latter varied with every experiment as was required according to the special subject.

If the solution got dry, fresh culture solution was often poured in. The culture was renewed every day at 6 P.M.

2. Chemical Analysis

Seedling samples were purposely harvested at the end of every experiment. Fresh weights were taken after blotting the excess water from the shoots and roots.

To determine the dry weights, the samples were put in the drying oven at 65°C to 70°C for three days and then their weights were taken.

Total nitrogen and protein nitrogen were determined from the dried tissue by the usual salicylic acid modification of the Kjedahl method. Asparagine nitrogen was obtained with the method of Vickery (1935).

3. Paper Chromatography

These methods were taken after the patterns employed by Steward and Thompson (1950).

Some 80 per cent cold alcohol was used to extract the soluble-nitrogen fractions. The extract was evaporated by pumping and redissolved in 1 ml. of water prior to chromatographic examination. A small amount of chloroform was added to remove certain fatty materials. The samples were examined with two-directional paper chromatography on sheets of Tung Young No. 2 paper, with phenol-water in the first direction and butanol-water in the second direction.

Experimentation and Results

FIRST EXPERIMENT The Formation of Asparagine in seedlings of Lupine germinating in the dark and in light.

PROCEDURE.—The lupine seeds that had been sterilized and soaked were germinating in the dark and in light separately, from 3 P.M. March 4 to 3 P.M. March 18, 1958.

The "light" series were kept in a greenhouse with air temperatures varying from 15°C to 35°C, subjected to the usual diurnal fluctuation of light intensity. The "dark" series were kept in a dark room, at the temperatures of about 20°C to 25°C. At 3 P.M. of the 3rd, 6th, 9th, 12th and 15th days seedlings of both series were harvested respectively. Twenty seedlings were chosen at random from each series, and their arithmetical mean in length were calculated.

RESULTS.—The seedlings of lupine germinated in darkness were healthy and vigorous, with exception at the last stage. After 15 days, the seedlings had undergone an obvious internal collapse. When harvested, the seedlings smelled of ammonia.

Table I shows that more asparagine was produced during the first 12 days of germination in darkness. This may be related to some what greater protein hydrolysis during this time and to the fact that protein hydrolysis was more complete in darkness.

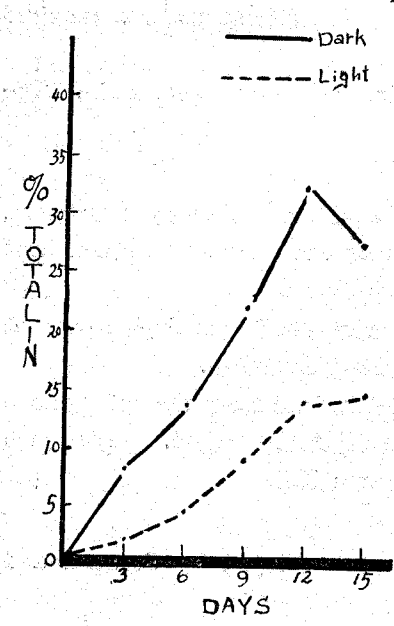

Fig. 1. Asparagine formation is favored by darkness in the stages of germination.

In the dark series, the asparagine is continually increasing in the seedlings all the stages of the germination. At the expiration of 12 days, it formed 53 per cent of water-soluble nitrogen and a third of protein had been transformed into asparagine.

The distribution of alcohol-soluble amino acids on the chromatogram is shown in Fig. 3. It indicates that the seedlings germinated in after darkness 12 days, accumulated a considerable contents of asparagine in accordance with the data by quantitative analysis.

Table 1. Nitrogen Compounds of Seedlings of Lupine Germinated in Darkness and in Light

Days	Origin	Dark					Light				
	0	3	6	9	12	15	3	6	9	12	15
Total-N mg/gm dry wt.	75.45	69.23	66.61	64.83	59.27	45.50	70.25	67.55	64.91	60.45	60.93
Water-soluble-N	12.15	21.05	30.54	37.56	36.92	28.35	21.25	24.69	27.35	29.00	31.78
Protein-N	63.30	48.18	36.07	27.27	22.35	17.15	49.00	42.86	37.56	31.45	29.15
Asparagine-N	trace	5.66	9.16	14.22	19.70	12.48	trace	3.32	6.22	8.56	9.04
Asparagine-N % Total-N	—	8.17	13.57	21.93	33.32	27.42	—	4.76	9.58	14.16	14.32
Asparagine-N % Water-soluble	—	26.88	29.99	37.86	53.56	42.02	—	13.04	22.70	29.70	28.44

Table 2. Determination of Amino Acids by Visual Observation of Lupine Seedlings Germinated in Dark and in Light After 12 Days

Amino acids	Dark	Light
Aspartic acid	++++	+++
Glutamic acid	+++	++
Serine	+	++
Asparagine	++++++++++++++	+++
Threonine	++	++
Alanine	++	++
Glutamine	+	+
Lysine	+++	++
Arginine	+++	++
Valine	+++++	+++++
Leucines	+++	+++
Phenylalanine	++++	+++

Minus (−) represents the disappearance of amino acid content.
Plus (+) indicates the amino acid content which tended to increase with increasing plus number.

SECOND EXPERIMENT Influence of Glucose on Asparagine Formation.

PROCEDURE.—The lupine seeds that had been treated as previously described were put to germinate in a dilute glucose solution (2%) in a dark room. The controlled series were germinating in the distilled water. This experiment began at 3 P.M. April 15, 1958.

After 12 days, on April 27, when the lupine seedling had accumulated a maximal concentration of asparagine nitrogen, the seedlings of both series were harvested, respectively.

Two grams of the seedlings of each series had been taken for paper chromatography by the methods as usual procedure.

RESULTS.—The lupine seedlings germinated on glucose solution appeared a shorter length in comparison with the controlled series.

The most striking feature of the nitrogen composition of lupine seedlings germinated with glucose is the high concentration of protein nitrogen. It forms about 56 per cent of the total nitrogen.

Table 3 shows that the formation of asparagine is inhibited by the presence of glucose. When seedlings germinated with glucose, asparagine forms 20 per cent of the total nitrogen, but in controlled series, it forms 36 per cent.

The distribution of alcohol-soluble amino acids on the chromatogram is shown in Fig. 5 and Fig. 6, and the spots are also identified in same figure. Exception asparagine, in controlled series, there are some high content of amino acids which are glutamic acid, serine, alanine, valine, and leucines.

Table 3. Length, Weights, Percentage of Moisture and Nitrogen Compounds of Lupine Seedlings Germinated With Glucose After 12 Days

Subject	With glucose	Without glucose
Root (cm)	8.41	10.85
Shoot	9.12	11.27
Total	17.53	22.27
*Fresh weight	105.16	113.76
*Dry weight	9.85	8.94
% Moisture	90.62	92.53
Total-N mg/gm dry wt.	59.41	55.52
Protein-N	33.74	23.19
Water-soluble-N	25.67	32.33
Asparagine-N	12.43	20.04
Asparagine-N in % total-N	20.92	36.07
Asparagine-N in % Water-soluble-N	48.42	58.23
Protein-N in % Total-N	56.79	41.62

* gm per 100 seedlings

Table 5. Determination of Amino Acids by Visual Observation of Lupine Seedlings Germinated With and Without Glucose After 12 Days

Amino acids	With glucose	Without glusose
Aspartic acid	++++	++
Glutamic acid	+++++	+++++
Serine	++	++++
Asparagine	++++	++++++++++++++
Threonine	−−	++
Alanine	++	+++++
Glutamine	−−	+
Lysine	+	+++
Arginine	−−	+++
Valine	++++	+++++
Leucines	−−	+++++
Phenylalanine	+++	+++++

Minus (−) represents the disappearance of amino acid content.
Plus (+) indicates the amino acid contents which tended to increase with increasing plus number.

THIRD EXPERIMENT Influence of Oxygen on the Formation of Asparagine.

APPARATUS FOR ANAEROBIC CULTURE—A suitable form of apparatus of anaerobic culture is shown in Fig. 8. The two bottles A and B are filled with saturated solution of meto (P-methylaminopheno Sulfate), dissolved in

20% NaOH, which serves to remove oxygen from the current of air. Bottle C is filled with the solution of hydroquinone, in 15% NaOH. It acts as an indicator to show whether the oxygen has been absorbed or not. The desiccator D is used to germinate the seeds of lupine under anaerobic condition.

The last vessels in the train are bottles E and F, filled with the solution of meto, which act as safety bottles to prevent the oxygen from coming back through the aspirator. The bottle F is connected to the aspirator pump with heavy walled tubing. A clamp serves to regulate the gas flow.

PROCEDURE.—From 3 P.M. May 2, 1958. The lupine seedlings which had been germinating for three days were placed in the desiccator under anaerobic condition in the dark room for continual germination.

Though the absorption train was made to be gas-tight, the current of air must be aspirated through the apparatus for 15 minutes three times a day.

The absorption solution of the train had been changed every other day in order to maintain a high efficiency in the absorption.

The controlled series were usually placed under aerobic condition in the same dark room. After a week, on May 9, the seedlings were harvested and treated just as the former experiment.

RESULTS.—Fig. 5 shows that seedlings germinated under anaerobic condition is shorter than those under aerobic condition, but they have a higher average dry weight.

Table 5. Length, Weights, Percentage of Moisture and Nitrogen Compounds of Lupine Seedlings Germinated in Aerobic and Anaerobic condition After 10 Days

Subject	Aerobic	Anaerobic
Roots (cm)	11.14	5.68
Shoot	10.32	4.73
Total	21.46	10.41
*Fresh weight	90.15	68.78
*Dry weight	6.76	7.95
% Moisture	90.11	87.58
Total-N (mg/gm dry wt.)	59.33	50.19
Protein-N	25.41	22.31
Water-soluble-N	33.92	27.88
Asparagine-N	14.32	8.76
Asparagine-N in % Total-N	24.13	17.47
Asparagine-N in % Water-soluble-N	42.21	31.26

* gm per 100 seedlings.

The data in Table 5 show that a considerable loss of nitrogen from the

seedlings germinated under anaerobic condition. In both series the hydrolysis of protein occurred at the same rapid rate.

In aerobic series, asparagine-N forms 42 per cent of the water-soluble nitrogen or 24 per cent of total nitrogen. In the other series, it forms 31 per cent and 17 per cent.

The distribution of alcohol-soluble amino acids on the chromatogram is shown in Fig. 10 and Fig. 11.

It is a very interesting fact that there are much amount of aspartic acid and arginine found in anaerobic series, so we may suggest that aspartic acid and arginine have relation to the precursor of asparagine.

Table 6. Determination of Amino Acids by Visual Observation of Lupine Seedlings Germinated in Aerobic and Anaerobic Condition

Amino acids	Aerobic	Anaerobic
Aspartic acid	++	+++++++++
Glutamic acid	++++	++
Serine	−−	++
Asparagine	++++++++++++++	++++
Threonine	++	++
Alanine	++++	++
Glutamine	−−	−−
Lysine	++	++
Arginine	++++	+++++
Valine	+++++	+++
Leucines	+++++	+++
Phenylalanine	+++++	+++

Minus (−) represents the disappearance of amino acid content.
Plus (+) indicates the amino acid contents which tended to increase with increasing plus number.

FOURTH EXPERIMENT The Formation of Asparagine through Different Nitrogen Supplies.

PROCEDURE.—Lupine seeds that had been sterilized and soaked were made to germinate in distilled water in a greenhouse, beginning from 3 P.M. September 27, 1958.

After a week, the seedlings were about 8 cm in height and then were divided in four groups to be cultured in the following solutions:

(1) Distilled Water
(2) Urea (0.2%)
(3) Sodium Nitrate (0.2%)
(4) Ammonium Chloride (0.2%)

In ammonium chloride solution, a little of $CaCO_3$ had been added, in order to neutralize the physiological acidity of ammonium salts. The legumes in particular seem to require calcium to neutralize the acid reaction of ammonium chloride, which appears to inhibit the formation of asparagine (Prianischnikov 1922). All the solutions were adjusted to pH 6.7 by adding 0.5–0.7 cc. of 1 N potassium hydroxide per liter.

RESULTS.—Seedlings grown in the urea solution show a more luxuriant than either of the groups of plants grown in the ammonium solution or in nitrate solution.

The influence of the forms of nitrogen in the culture solution on the forms of nitrogen in the seedlings is evident in Table 7.

The total nitrogen in the ammonium seedlings is the greatest amoung the four groups. This is due to a much greater concentration of insoluble or protein nitrogen. Appreciable amount of the total nitrogen is also found in the urea seedlings.

Seedlings cultured in the ammonium solution are characterized by larger quantities of asparagine. Asparagine nitrogen is also formed a relatively high proportion of the total nitrogen present in urea seedlings.

Table 7. Weight, Percentage of Moisture, and Nitrogen Compounds of Lupine Seedlings Cultured on Different Nitrogen Supplies After 21 Days

Subjects	Distilled Water	Urea	Sodium nitrate	Ammonium chloride
*Fresh weight	71.36	68.74	68.15	70.64
*Dry weight	6.84	7.12	6.80	7.25
% Moisture	90.14	89.63	90.02	89.73
Total-N	54.75	76.46	65.03	78.66
Protein-N	23.96	35.16	31.05	34.05
Asparagine-N	10.42	18.04	12.53	20.16
Asparagine-N in % Total-N	19.03	23.59	19.26	25.63
Water-soluble-N	30.79	41.30	33.98	44.61

* gm per 100 seedlings.

Discussion

From the data of these experiments, we have found that there are five significant points that constitute a considerable importance to the formation of asparagine and its physiological function in the lupine seedlings.

1. The similarity of the catabolic process in the lupine seedlings whether kept in light or in the dark.

From the Table 4, we know very well that the protein hydrolysis proceeded at approximately the same rate regardless of illumination for the first six days; subsequently, after 12 days, the rate of hydrolysis of protein in the "light" seedlings diminished, while the rate in the "dark" seedlings still maintained.

This dominating process of nitrogen metabolism in the germinating seeds is, as suggested by many plant physiologists, the hydrolysis of the reserve protein of seeds and then the protein is converted into amino acids. These amino acids are then believed to be transported to the growing embryonic regions where they are resynthesized as protein.

However, the lupine seeds contained a low amount of carbohydrate. In etiolated seedlings, under starvation condition, amino acids may be used as substrates for respiration. At the end of oxidation, an excess of ammonia was produced from the seedlings (Prianischnikov 1922). According to Prianischnikov's hypothesis, asparagine synthesized in seedlings is in response to an excess of ammonia, and provides a mechanism whereby the ammonia concentration is ordinarily maintained below a toxic level.

Schulze (1919) found that the amount of asparagine accumulated greatly exceeds the amount already contained in the reserve protein of the seeds and that it is released by protein hydrolysis during the germination. We are shown clearly that asparagine synthesis must take place at the expense of other amino acids.

Fig. 1 and Table 1 show that during the first 6 days of germination in darkness more asparagine and water-soluble nitrogen was produced than that in light. This may be related to the somewhat greater protein hydrolysis during this period in darkness than in light and to the fact that protein hydrolysis was more complete in darkness. The figures in Table I show that after 15 days a three-fourth of protein nitrogen had been converted into water-soluble nitrogen in darkness, whereas in light, only half protein nitrogen could be water-soluble.

Vickery (1943) reported that the synthesis and accumulation of asparagine is characteristic of the germination and growth of etiolated seedlings only over a restricted period. With advancing depletion of the reserve of the seedlings, the asparagine finally begins to disappear, while free ammonia appears simultaneously. This state is ordinarily followed very quickly by the death of plant.

2. The influence of carbohydrates on asparagine formation.

Table I shows that in darkness asparagine synthesis was very rapid during the 6–12 days period. At the end of the 12th day, the seedlings contained more than twice as much asparagine as that in the seedlings germinating in light. Apparently it may be suggested that metabolism conditioned by the presence

or absence of light is of primary importance in the secondary synthesis of asparagine. This explanation seems not accurate. Lupines growing in light have no such accumulation of asparagine as that found in lupines growing in dark. It is not due to the direct effect of light but to the presence of carbohydrates in light (Prianischnikov 1922). In Table 3 it is evident that lupine seedlings germinating with glucose accumulated a little asparagine.

One may conclude as did Prianischnikov (1922), therefore, that, because of carbohydrate shortage, reserve proteins of seeds will be hydrolyzed and there will be found amino acids, which, through oxidation and secondary synthesis, will produce acid amides, asparagine. But if carbohydrate supply is abundant, the reverse process will take place.

3. The importance of relation of the asparagine formation with oxidation.

Table 5 shows evidently that protein may be decomposed in lupine seeds with or without the presence of oxygen. In the presence of oxygen, however, asparagine appears as the main water-soluble nitrogen substance. With further oxidation it is broken down to NH_3. That oxidation is really essential to the production of asparagine has been demonstrated by Suzuki (1902) with barley and soybeans. The latter found that there was not only an increase of asparagine when seedlings were germinating in darkness with the presence of oxygen, but also a marked decrease of amino acid. Without the supply of oxygen, asparagine is not formed but amino acids are accumulated. With further oxidation and absence of carbohydrates, ammonia is produced in large quantities owing to the break-down (oxidation) of asparagine.

Sure (1916) also showed that in the shoots of pea seedlings there was a similar decrease of amino acid and ammonia. This indicates that "amino acid serves or asparagine production in the nitrogen metabolism of the etiolated pea plants".

What kind of amino acid serves for the formation of asparagine through the process of oxidation is still unknown at the present time. According to Murneek (1935), the mono-amino acids may be oxidized and release amino groups (NH_2). Some of these may be oxidized to aspartic acid, and others will split off with ammonia. A union of aspartic acid with ammonia forms ammonium salts, from which, through dehydration, asparagine is produced.

The figures in Table 6 show that there is a great amount of aspartic acid and arginine bear relation to the precursor of asparagine.

4. Asparagine being synthesized from ammonium salts.

Table 7 indicates that ammonium chloride is a better nutrient form of nitrogen for the formation of asparagine than nitrate. In other words, this experiment shows evidently that the conversion mechanism of ammonium nitrogen to asparagine nitrogen is easily obtained but it is not so with nitrate.

Asparagine nitrogen may be converted into protein nitrogen under abundance of carbohydrate.

Prianischnikov (1922) has demonstrated that by supplying salts of nitrogen to certain seedlings their asparagine content is markedly increased. For this purpose ammonia is a better source of nitrogen than nitrates, when the physiological acidity of ammonium salts is neutralized by a base. The importance of calcium in this respect is not clearly understood (Murneek 1935). Prianischnikov thinks that Ca increases respiration and hydrolysis of protein.

Except ammonium chloride, Table 7 shows that urea is also a better nutrient form for the formation of asparagine. According to Suzuki (1902), it is frequently a better nutrient form of nitrogen for the formation of asparagine than ammonium salts.

5. The mechanism of asparagine formation in plants.

Although the close correlation of asparagine synthesis with the presence of ammonia is obvious, the nature of the other components and the process of synthesis are by no means clear. That asparagine may rise directly from the tissue protein by hydrolysis of peptide bone is possible but the quantities actually observed under many experimental conditions are far in excess of the quantities that could be provided from this source on any resonable hypothesis of protein constitution. This was clearly pointed out by Schulze (1911) many years ago.

The probability that precursors of asparagine are products of carbohydrates metabolism follows from the early work of Suzuki (1902) and was carefully examined by Prianischnikov (1922) and by Smirnov (1923) but experiments designed to ascertain their nature have not been entirely sucessful.

Asparagine 在羽扇豆幼苗體內之形成及其生理意義

楊　冠　政

爲探討影響 Asparagine 在羽扇豆幼苗體內形成之各項環境因子，以及 Asparagine 之形成與各種氨酸間之關係乃舉行本實驗。將黃花羽扇豆 (Lupinus luteus) 置於光與暗，缺氧，葡萄糖液，和各種含氮化合物溶液中萌發，經一定時期後分析其氮素成分，並用濾紙色層分析法 (Paper Chromatography) 分析其氨酸之種類與含量。

在暗中萌發之幼苗，其 Asparagine 之含量顯然日漸增加，十二天後 Asparagine 氮占全氮量之 33.32%，而在光中萌發之幼苗爲 14.16%。故光爲 Asparagine 形成之一抑止因子。

葡萄糖液 (2%) 中萌發之幼苗，其體內形成之 Asparagine 大爲減少。萌發十二天之幼苗，其 Asparagine 氮僅占全氮量之 20.92%。其氨酸之種類亦減少，而 Aspartic acid, glutamic acid, valine 等氨酸之含量却增加。

氧氣缺乏狀態下，幼苗中之全氮量及 Asparagine 氮均告減少。全氮量之減少係由於

氣態 Ammonia 之消失。Asparagine 之形成顯然與氧化作用 (Oxidation) 有關。何種氨酸經氧化而成 Asparagine，迄今仍爲懸案；而在缺氧狀態下，氨酸 Arginine 積聚甚多，此事實有進一步探討之價值。

尿素，硝酸鈉，氯化銨等含氮化合物供給幼苗萌發時，尿素與氯化銨均有助於 Asparagine 之形成。

綜觀各實驗，得知凡含 Asparagine 氮較多之幼苗，其植株均有徒長 (Elongation) 之現象。Asparagine 與植物細胞生長是否有密切關係，實有研究之必要。(摘要)

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