ACTA CHEMICA SCANDINAVICA 2 (1948) 127—139

On the Formation of Amino Acids and Amides in Green Plants

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The synthesis of amino acids and proteins in green plants during the assimilation of different nitrogen compounds forms one of the most important links in the chain of the nitrogen cycle in nature. This phenomenon is very slow under normal conditions owing to many limiting factors. In experimental conditions we have, however, the opportunity of eliminating these limiting factors and arranging the experiments so that all processes in cells occur very intensively. We can then follow with chemical analyses the formation and transformation of different intermediate compounds. In this connection, however, it must be emphasized, that obtaining a detailed picture of the mechanism of these processes demands that the experimental time even in plant experiments must be very short, only some hours.

The work reported here was undertaken to investigate more intimately and quantitatively the mechanism and different phases of amino acid synthesis in green plants.

One of the central problems in the study of nitrogen assimilation in the living cells in general is the formation of the primary amino acids. The investigations of recent years emphasize the primary and central position of aminodicarboxylic acids, glutamic and aspartic acids, in this process. In this work particular attention thus has been paid to these amino acids and also to alanine which can easily be formed from the aminodicarboxylic acids, for example through the transamination reaction.

EXPERIMENTAL

All experiments were carried out with pea plant *(Pisum sativum sp.)*. This because of its fairly rapid growth and lively nitrogen metabolism. Moreover, it can develope inoculated with the specific nodule organisms without any combined nitrogen. It is

therefore possible to obtain plant material which does not contain a disturbing amount of inorganic (ammonia or nitrate) nitrogen. For practical reasons all the plant material was grown in the greenhouse in water culture inoculated with an effective *Rhizobium* strain. After a growth period varying from 4 to 5 weeks when the plants were in a very high degree of metabolic activity they were harvested for experiments. For this purpose they were cut just above the seed and the tops were placed in glass beakers containing a neutral aqueous solution of the nitrogen compound to be tested. The beakers were placed in a very intensive electric light. The suction due to transpiration caused a rapid rise of liquid into the plants and the transformation of the nitrogen taken up began immediately. During the experiment the chemical changes occurring as a result of the nitrogen uptake were followed at intervals of some hours with analyses. The whole experimental time was at the most 24 hours. Signs of wilting were observed only by feeding with ammonium salt. Thus it seems probable that the synthetical processes will take place as do the corresponding processes in the normal intact plant.

Analytical methods

For the analyses the cell sap was expressed from an equal number of plants at different points of the experiment. When *ammonia and the organic nitrogen fractions* were analysed the sap was deproteinized with trichloracetic acid. When again *nitrate* and *nitrite* were analysed lead acetate was used.

The analyses of the different nitrogen fractions were performed by the following methods. *Total organic nitrogen* was determined by the Kjeldahl micro method. If there was ammonia present the amount of ammonia nitrogen was subtracted from the Kjeldahl nitrogen. *Amino nitrogen* was determined after amide hydrolysis and removal of ammonia by the van Slyke volumetric method. *Ammonia nitrogen* was distilled according to Pucher *et al* [1]. *Total amide nitrogen* was likevise determined according to Pucher *et al.* *Asparagine* and *glutamine* were differentiated according to Schwab [2]. *Total nitrogen of amides* was calculated by multiplication of the corresponding amido nitrogen by two. *Aminodicarboxylic acids* were precipitated according to the principle of Foreman. The precipitate was dissolved in sulphuric acid. From this solution *aspartic acid* was determined by the malic acid method of Pucher *et al.*[3] adapted by Arhimo [4] to aspartic acid. Prior to the determination, the aliquot was extracted with ether. *Glutamic acid nitrogen* was calculated by subtracting the aspartic acid nitrogen from the total Foreman nitrogen. *Alanine* was determined by the method of Roine and Rautanen [5] after it had been oxidized with ninhydrin according to Virtanen and Rautanen [6]. *Nitrate* determination was carried out as described by Burström [7]. *Nitrite* was determined according to Jendrassik and Falcsik-Szabo [8]. *Malic acid* was extracted with ether in a Partheil-Rose extractor and then determined according to Pucher *et al.*[3]

Results

All results are given in milligrams nitrogen per 10 ml of original plant sap.

The first subject of this work was to follow the utilization of inorganic nitrogen compounds (ammonia and nitrate). In feeding solutions, ammonium

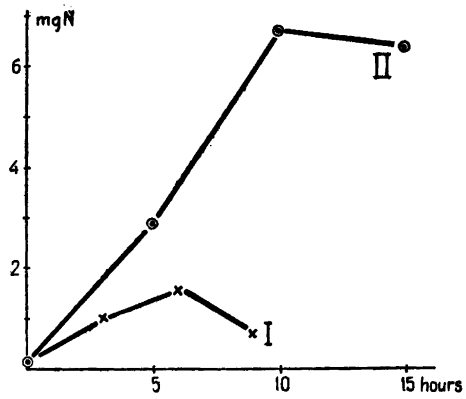

Fig. 1. The rate of the accumulation and disappearance of ammonia and nitrate nitrogen in plant sap during and after the uptake of corresponding nitrogen compound.

Curve I: Ammonia N.
» II: Nitrate N.

sulphate and calcium nitrate were used in concentration of 0.9 mg N per 1 ml of solution.

The objective in a preliminary experiment was to follow the rise and accumulation of ammonia and nitrate nitrogen in plants, as well as to state how rapidly they will be used for synthesis of organic nitrogen. For this purpose the accumulation of the corresponding nitrogen in the plants was followed with analyses during 6 hours in ammonia solution and during 10 hours in nitrate solution. Then the ammonia plants were transferred for 3 hours and the nitrate plants for 5 hours to water and the disappearance of the corresponding nitrogen was determined. Results are revealed in Fig. 1.

Although the results owing to the different times of the experiments are not quite comparable with each other it can be noted that ammonia nitrogen accumulates in plants much more slowly and disappears more rapidly than nitrate nitrogen. This fact indicates that at least the primary utilization of ammonia nitrogen is more rapid than the utilization of nitrate nitrogen.

The first stage of the utilization of nitrate is reduction. This process can occur in the roots as well as in the green parts of plants (*cf.* Dittrich [9], Burström [10]). The course of the reduction in plants was estimated during the uptake of nitrate by analyzing nitrate and its reduction products nitrite and ammonia. A control experiment was also made in which the plants were placed in water without any nitrogen sources. In them only ammonia was determined. Results are given in Table 1.

On the basis of the data in Table 1 it can be stated that during the continuous accumulation of nitrate only very little nitrite appears. The amount of nitrite after having reached rapidly its maximum concentration decreases a little or remains on the same level. Although the amount of ammonia even

Table 1. Amounts of nitrate and its reduction products in nitrate and control plants at different points of experiment.

		Hours			
		0	5	10	15
Nitrate plants:	Nitrate N	0.20	1.36	1.92	2.31
	Nitrite N	0.0003	0.0021	0.0019	0.0018
	Ammonia N	0.062	0.167	0.194	0.090
Control plants:	Ammonia N	0.062	0.055	0.075	0.068

in the nitrate plants is relatively small it can, however, be stated that some ammonia has been formed in nitrate plants but not in control plants. The appearance of ammonia in nitrate plants is of a temporary nature because it falls after some hours to the initial low level.

The possibility that hydroxylamine can act as an active intermediate compound in nitrate and ammonia assimilation was first suggested in 1884 by Meyer and Schulze [11]. Later the formation of oxime nitrogen as well in the assimilation of atmospheric nitrogen (Endres [12] with *Azotobacter*, Virtanen and Laine [13] in root nodules) as in the assimilation of nitrate nitrogen (Endres [14] with *Azotobacter*, Virtanen and Arhimo [15] in plants, Virtanen and Csáky [16] with yeast) has been established.

For the estimation of hydroxylamine and oxime, extraction with trichloracetic acid was used. It is impossible, however, to obtain colourless solutions from the green parts of plants when this agent is used. Hydroxylamine and oxime were therefore tested only in root extracts. *Hydroxylamine* was determined by the technique of Blom [17] and *oxime* according to Endres [12].

Four experiments were made in which the intact plants were kept in nitrate solution from 3 to 11 hours. All the hydroxylamine tests gave negative results. In two experiments could be noted a reaction of oxime which can be interpreted as positive.

The first task when beginning to follow the syntheses of organic nitrogen compounds due to the external supply of nitrogen was to determine the changes occurring in plant sap without any uptake of nitrogen. For this purpose the plants were placed in water and at certain intervals samples were taken for analysis. Results are given in Table 2.

It is evident that the composition of organic nitrogen fractions changes to a certain degree during the experimental time. The greatest variations can be observed in the fractions of glutamic acid and glutamine. Moreover, the inequality of the results is chiefly caused by the unhomogeneity of the analyzed plant samples.

Table 2. Amounts of organic nitrogen compounds during the experiment without any uptake of nitrogen.

	Hours			
	0	5	10	14
Ammonia N	0.12	0.05	0.12	0.07
Glutamic acid N	3.19	2.86	3.55	3.74
Aspartic acid N	1.41	1.39	1.33	1.33
Total N of glutamine	0.28	0.28	0.40	0.24
Total N of asparagine	7.86	7.52	8.00	7.82
Total N of both amides	8.14	7.80	8.40	8.06

During the uptake of nitrate the analyses gave the results shown in Table 3. The differences which can be observed in this case are of the same order as in the control experiment described above. The feeding of nitrate nitrogen has thus not caused any significant changes in the analyzed organic fractions of plant sap.

On the other hand, by feeding with ammonia nitrogen quite a different picture of the distribution of the soluble nitrogen compounds was observed. In order to give a clear picture of the relations existing between the alterations of different compounds the results are illustrated graphically in Fig. 2.

Immediately after the uptake of ammonia had begun intensive synthetical processes could be noted. It will be seen from the curves that glutamic acid and its amide, glutamine, are accumulated abundantly. The amount of aspartic acid increases too but its increase is much smaller and can be observed only in the form of the amide, asparagine. The accumulation of alanine is very slight and its amount decreases later. The pea plant used in these experiments is a typical asparagine plant (*cf.* Schwab [2]) which is seen from the relation of both amides. In this case for example over 95 % of the total amount of amide nitrogen was in the beginning asparagine nitrogen, the amide synthesized first to a greater extent during the uptake of ammonia was, how-

Table 3. Amounts of organic nitrogen compounds during the uptake of nitrate.

	Hours			
	0	5	10	15
Ammonia N	0.03	0.09	0.09	0.07
Glutamic acid N	4.02	3.61	3.59	5.17
Aspartic acid N	0.98	0.98	1.04	0.95
Total N of both amides	4.58	4.06	4.06	3.44

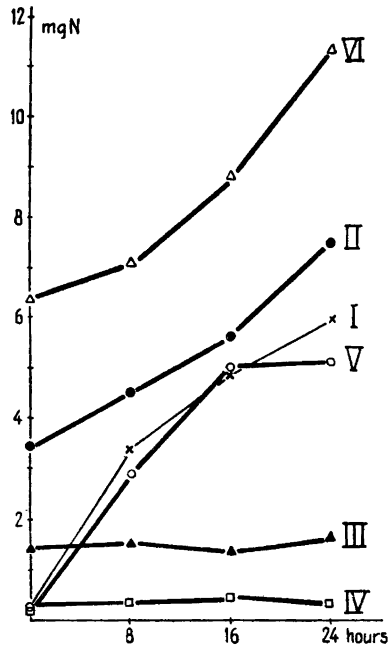

Fig. 2. Amounts of organic nitrogen compounds during the uptake of ammonia.

Curve I: Ammonia N.
» II: Glutamic acid N.
» III: Aspartic acid N.
» IV: Alanine N.
» V: Total N of glutamine.
» VI: Total N of asparagine.

ever, glutamine. The increase of asparagine takes place slowly in the beginning but continues, even accelerating, although the formation of glutamine has already come to a standstill. It can be seen from the curves of both amides that there is a correlation between them.

On the basis of the data given in Fig. 2 the amount of dicarboxylic acid nitrogen, the total nitrogen of amides, and alanine nitrogen are summed up at the different points of the experiment and illustrated graphically in Fig. 3. In the same graph is also illustrated the total nitrogen of plant sap at the corresponding moments.

It can clearly be seen from these curves that the increase of the fractions summed up above suffices to cover completely the increase of total nitrogen of plant sap. The accumulation of alanine is, however, so small that it is of no great importance. The inconsistency that the absolute values of total nitrogen are smaller than the sum of the partial fractions is apparently due to the small errors in analyzing of the many partial fractions. In spite of these errors it is evident that the plant sap may contain at most quite minute amounts of other nitrogen compounds. All these constituents accumulated in plant tissue during the primary stage of the ammonia assimilation are directly or indirectly dependant on each other.

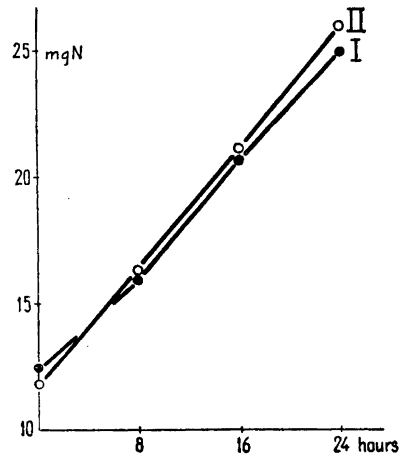

Fig. 3. Total organic nitrogen, and the sum of the nitrogen of different organic fractions during the uptake of ammonia.

Curve I: Total organic N.
» II: Sum of fractions of glutamic acid N, aspartic acid N, total N of amides, and alanine N.

The final aim of the nitrogen assimilation is to build up the cell protein. The amino acids are used to this protein synthesis. The disappearance of the amino nitrogen from the cell sap may be considered as a measure of the protein synthesis. To elucidate this fact an experiment was arranged in which the plants were first placed in ammonia solution for 14 hours and then transferred to water for 5 hours. The changes in ammonia nitrogen and total amino nitrogen were followed. When calculating the results, it was taken into consideration that the organic nitrogen of amido groups was distilled off before the determination of amino nitrogen according to van Slyke. The results of this experiment are given in Table 4.

Table 4. Variations of amounts of ammonia and total amino nitrogen during and after the uptake of ammonia. Plants were kept first 14 hours in ammonia solution and then 5 hours in water.

	Hours		
	0	14	19
Ammonia N	0.31	3.31	1.59
Total amino N	9.11	12.36	11.80

When the external supply of ammonia was interrupted the amino nitrogen of sap diminished although the sap still contained considerable amounts of ammonia from which it continuously synthesized new amino nitrogen. The protein synthesis thus takes place simultaneously with the processes described above. According to this it is very interesting to note that during the intensive

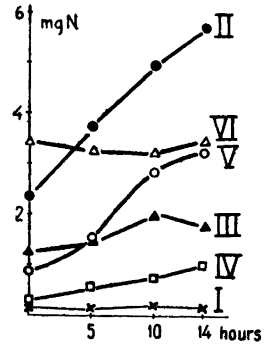

Fig. 4. Amounts of organic nitrogen compounds during the uptake of glutamic acid.

Curve I: Ammonia N.
» II: Glutamic acid N.
» III: Aspartic acid N.
» IV: Alanine N.
» V: Total N of glutamine.
» VI: Total N of asparagine.

formation of amino acids and protein, dicarboxylic acid and amido nitrogen almost exclusively were accumulated in plant sap. In this work therefore when continuing the experiments, attention was paid to the more minute investigation of the relations between aminodicarboxylic acids and amides. For this purpose the plants were fed with glutamic and aspartic acids and alanine. The concentration of the feeding solutions was in every experiment 0.5 mg N per 1 ml of solution. The analytical data of these experiments are illustrated in Figs. 4, 5, and 6.

It will be seen from the curves that during the uptake of glutamic and aspartic acid the accumulation of corresponding aminodicarboxylic acids in plant sap is very slight. This is due to the very rapid conversion of these acids not only into each other but into amides and alanine. On the other hand, the increasing of alanine in plant sap when feeding with this amino acid can be clearly noted at least at the beginning. In this case only the amount of glutamic acid at the first phase increases a little but the total amount of amides decreases during the whole experiment. The appearance of free ammonia is in every case very small and its amount does not increase during the experi-

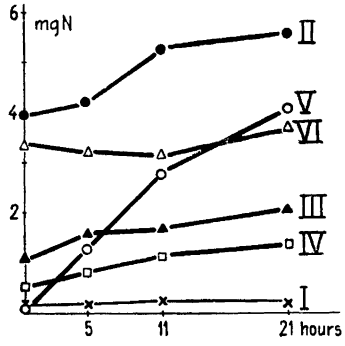

Fig. 5. Amounts of organic nitrogen compounds during the uptake of aspartic acid.

Curve I: Ammonia N.
» II: Glutamic acid N.
» III: Aspartic acid N.
» IV: Alanine N.
» V: Total N of glutamine.
» VI: Total N of asparagine.

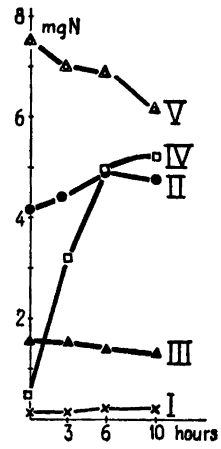

Fig. 6. Amounts of organic nitrogen compounds during the uptake of alanine.

Curve I: Ammonia N.
» II: Glutamic acid N.
» III: Aspartic acid N.
» IV: Alanine N.
» V: Total N of both amides.

ment. The splitting of amino group as ammonia must, however, have occurred because such considerable amounts of amides are formed. It is of particular interest to note that the amide primarily formed both from glutamic as well as aspartic acid is glutamine.

These experiments make evident that aspartic acid also is in fact a very active compound, though in the experiments with ammonia it seemed to be more stable than glutamic acid. Thus we can suppose that it could not accumulate in the earlier experiments because it changes so rapidly. Consequently, the question of the primary amino acid is very difficult to solve, on the basis of these experiments alone.

The key position of aminodicarboxylic acids and alanine in amino acid synthesis is partly explained by the central importance of the corresponding α-keto acids in the carbohydrate metabolism. They are members of the tricarboxylic acid cycle of Krebs [18]. Malic acid which is so characteristic of plants is also included in this cycle. All these compounds of the cycle are closely connected with each other as well as with the amino acid and protein metabolism. In order to obtain some information of the proportions between these carbohydrate derivatives and the nitrogen metabolism, only malic acid as a representative of carbohydrate derivatives was analyzed in experiments described in this paper, because the accurate quantitative determination of this compound is very easy. The amount of malic acid (given in mg per 10 ml of the original plant sap) at different points of experiments subtracted by the amount at zero point is illustrated in Fig. 7.

When the plants did not receive any nitrogen (control) a large quantity of malic acid was accumulated in plant tissue. The feeding of organic nitro-

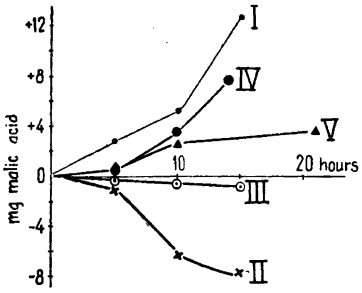

Fig. 7. Changes in the amounts of malic acid when plants were fed with different nitrogen compounds.

Curve I: Without any nitrogen source (control).
» II: With ammonia.
» III: » nitrate.
» IV: » glutamic acid.
» V: » aspartic acid.

gen in form of glutamic and aspartic acid also caused the increase in the amount of malic acid. On the other hand, when supplying inorganic (ammonia and nitrate) nitrogen, the disappearance of malic acid was observed. These findings are of some interest particularly because they show that even the carbon chain included in the organic nitrogen compounds is of importance in the assimilation of organic nitrogen.

DISCUSSION

According to our present knowledge ammonia is the central inorganic nitrogen compound in the nitrogen assimilation in the living cells in general. The temporary increase of ammonia in green plants even during the assimilation of nitrate, supports this view and at least that part of nitrate which has been reduced to ammonia is assimilated in this way. There may, however, be the possibility that even some other of the reduction products of nitrate *e. g.* hydroxylamine, can act as an active intermediate. The observations concerning the presence of hydroxylamine and its condensation product with keto bodies, oxime, during the nitrate assimilation make the existence of this possibility evident. The nature of this oxime and its real importance in nitrate assimilation are, however, quite obscure. According to Virtanen [19] it is possible that although in the fixation of atmospheric nitrogen by leguminous plants a formation of oxime can be noted this may be the result of a by-reaction, and the main reaction leads over ammonia.

On the basis of the results of this work the other primary organic nitrogen compounds which are synthesized during the nitrate assimilation are also of indefinite nature. So far as nitrate is reduced to ammonia the compounds which have been primarily synthesized are analogous with the compounds which are formed by the utilization of ammonia. The primary utilization of nitrate nitrogen is in any case much slower than the utilization of ammonia

nitrogen and on account of this, ammonia can not accumulate in plant sap to so great extent as nitrate. In agreement with this Virtanen and Linkola [20] have shown that in sterile cultures the intact plants have taken ammonia nitrogen more rapidly than nitrate nitrogen.

During the intensive uptake of ammonia there can be observed a very rapid and abundant accumulation of organic nitrogen in plant sap. This increase of the soluble nitrogen is formed exclusively by aminodicarboxylic acids, their amides, and alanine. It seems safe to conclude that these amino acids have a central position in the assimilation of different nitrogen compounds. The object of the formation of these amino compounds is to change rapidly the inorganic ammonia nitrogen into the organic form.

On account of the very easy interconversion of these amino acids it is difficult to solve the question of the primary amino acid. The synthesis of the other amino acids takes place more slowly and their synthesis is the limiting factor of the protein formation and thus only the above amino acids can accumulate in cells.

These results are in general agreement with the observations of Roine [21] made with yeast.

The amide problem which has had such a prominent position in the study of nitrogen metabolism in plants, comes out in this work in a quite new light. This question cannot be explained as a separate one but only in connection with the other problems of nitrogen metabolism because it is intimately connected with these primary processes of ammonia assimilation. In every case in which the amide formation could be observed independent of the source of nitrogen, the amide synthesized first was glutamine. The more stable amide to which also glutamine later was transformed was asparagine. When glutamic or aspartic acids were the sources of nitrogen there could be noted the synthesis of amides without formation of free ammonia. On the basis of this it is evident that even for plant cell it is in general very important to prevent the accumulation of ammonia. In this connection it is therefore necessary to emphasize that there seems to be no difference in principle between the mechanism of the amide formation and their importance on the one hand in plant and, on the other hand, in animal organisms. The quantitatively more important role of amides in plants is based on the peculiar nitrogen economy of these organisms in general.

The formation and transformation of all the nitrogen compounds to which attention has been paid in this work take place directly or indirectly through the wellknown reactions by which the amino or amido groups generally are formed in living cells. In these processes the metabolism of the carbon chains from which the amino acids are formed has generally been overlooked. As

we now know only the keto acids corresponding glutamic acid, aspartic acid, and alanine are members of the tricarboxylic acid cycle of Krebs, which is of so great importance in the carbohydrate metabolism. This fact explains partly the importance as well as the easy interconversion of these amino acids. The synthesis of the other amino acids necessary for the protein synthesis, on the other hand, and the final aim of the nitrogen assimilation, the protein formation, are still quite hypothetical.

SUMMARY

In this paper some observations concerning the formation of organic nitrogen compounds in green plants during the assimilation of inorganic and organic nitrogen compounds have been reported.

It was shown that nitrate is rapidly reduced to ammonia and thus can be assimilated in the same way as ammonia. On the other hand, there is the possibility that the nitrate assimilation can occur through hydroxylamine and oxime but this process is as yet quite obscure in its details.

During the uptake of ammonia there was noted a very rapid and intensive synthesis of organic nitrogen compounds in plant sap. This organic nitrogen was composed chiefly of aminodicarboxylic acids and their amides. In addition to these alanine also seems to be of importance in this process.

The metabolic relations between aminodicarboxylic acids, their amides and alanine were investigated in detail by feeding the plants with these amino acids. Their easy interconvertibility was observed.

The question of the primary amino acids is discussed.

The formation of amides and their importance in the nitrogen metabolism of plants was also investigated and is discussed.

The relations between carbohydrate and amino acid metabolisms was investigated using malic acid as a representative of the carbohydrate derivatives.

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Received January 16, 1948.