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Amino Acid Metabolism of Pea Leaves

DIURNAL CHANGES AND AMINO ACID SYNTHESIS FROM ^{15}N-NITRATE[1]

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ALFRED BAUER,[2] AILEEN A. URQUHART, AND KENNETH W. JOY[3]
Department of Biology, Carleton University, Ottawa, Canada K1S 5B6

ABSTRACT

In the young leaves of pea (*Pisum sativum* L.) plants, there was a diurnal variation in the levels of amino acids. In the light, total amino nitrogen increased for the first few hours, then stabilized; in the dark, there was a transient decrease followed by a gradual recovery. Asparagine, homoserine, alanine, and glutamine accounted for much of these changes. The incorporation of ^{15}N into various components of the young leaves was followed after supply of ^{15}N-nitrate. ^{15}N appeared most rapidly in ammonia, due to reduction in the leaf, and this process took place predominantly in the light. A large proportion of the primary assimilation took place through the amide group of glutamine, which became labeled and turned over rapidly; labeling of glutamic acid and alanine was also rapid. Asparagine (amide group) soon became labeled and showed considerable turnover. Slower incorporation and turnover were found for aspartic acid, γ-aminobutyric acid, and homoserine. Synthesis and turnover of all of the amino acids continued at a low rate in the dark. γ-Aminobutyric acid was the only compound found to label more rapidly in the dark than in the light.

During much of the life of the plant, the developing leaves constitute the major sink for nitrogen transported from roots. The nitrogen supply consists of nitrate together with a varying proportion of organic nitrogen, depending on the species and conditions (22). The amides glutamine and asparagine are usually predominant as transport and storage forms of nitrogen (22). In field peas (*Pisum arvense*), over 50% of the nitrogen in bleeding xylem sap can be organic, with the amides and homoserine as major components (24).

Glutamate dehydrogenase has been considered to play a major role in ammonia assimilation. It is present in many plant tissues and has been purified from pea roots (21); however, the enzyme has a low affinity for ammonia. Glutamine synthetase is present in plants, and recent work (7, 9, 19) indicates that it is located in chloroplasts; the affinity for ammonia may be up to 1,000-fold greater than that of glutamate dehydrogenase (20). Many reactions are known to involve glutamine, and the recent demonstration of the glutamate synthase reaction in plants (13) provides an attractive system both for utilization of transported glutamine, and (together with glutamine synthetase) for the primary assimilation of ammonia (review by Miflin, 17). However, the presence (or absence) of enzymes under assay conditions in extracts is not a sure indication of the role of the enzyme within the plant, nor can the relative flow of metabolites through alternate pathways be judged from enzyme activities.

Additional information on the flow of nitrogen through the pools of various components of primary nitrogen assimilation has been obtained from studies using the stable isotope ^{15}N. A detailed kinetic analysis of soluble nitrogen compounds in roots of rice seedlings supplied with $^{15}NH_4^+$ (29) or $^{15}NO_3^-$ (30) showed that most rapid incorporation was into glutamine, followed by glutamic acid then by aspartic acid and alanine. Amide and amino groups of glutamine were not analyzed separately. Canvin and Atkins (4) demonstrated a light-dependent incorporation of nitrogen from nitrate, nitrite, and ammonia into the amino acid fraction of leaves. Labeled nitrate supplied to cut shoots of *Datura* was converted predominantly to glutamine in photosynthesizing leaves (15). Detached shoots of *Pisum* utilized glutamine-amide nitrogen, -amino nitrogen, and nitrate equally well as nitrogen sources, and the amino acids of leaves and other shoot parts showed similar labeling patterns irrespective of the source (16).

There is a need for more detailed studies of the flow of nitrogen in the plant; questions remain about the relative importance of assimilatory pathways and the pathways of utilization of the various nitrogen sources arriving in the growing leaf. This paper describes the pea plant system chosen as experimental material for investigation of some of these questions, and the results of some first experiments using ^{15}N.

MATERIALS AND METHODS

Pisum sativum (L. cv. Little Marvel) seeds were surface-sterilized in sodium hypochlorite, rinsed, and then soaked in aerated H_2O for 12 hr. They were germinated in vermiculite for 5 days, then transferred to containers of aerated Hoagland-type nutrient solution (4 mM nitrate) which was replenished at weekly intervals. The plants were grown in cabinets with 12 hr photoperiod (1,300 ft-c, 27 C) and 12 hr dark (18 C). Cotyledons were removed 15 days after start of germination, when the plants had three expanded leaves. Plants were used about 20 days after start of germination, selecting those in which the fourth leaf was just fully opened and the fifth leaf was about half expanded. The young leaf tissue analyzed consisted of the fourth and fifth leaves (including internode, stipules, and petioles) and the apical bud. The tissue was frozen in liquid nitrogen, then soluble compounds were extracted overnight with 80% ethanol at 4 C (23). Samples were concentrated at 30 C, and an aqueous fraction (at pH 3.5) was obtained after partition against chloroform.

Xylem sap exuding from the roots was obtained by collecting the sap bleeding from stumps of detopped plants. Plants were severed at the lower epicotyl, and the cut end was rinsed and blotted dry. Sap was collected in a capillary from several actively exuding plants, over a period of 30 min.

When tissue or sap samples were taken during the dark period, plants were handled in dim green light.

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[2] Present address: Pflanzenphysiologisches Institut der Universitat, Altenbergrain 21, 3013 Bern, Switzerland.

[3] To whom reprint requests should be addressed.

Analysis. Free ammonia (3), nitrate (6), and total α-amino nitrogen (28) were estimated in soluble fractions. Quantitative analysis of amino acids was carried out with a Technicon analyzer, using a lithium buffer system. For preparative separation, extracts were first separated by high voltage electrophoresis on Whatman 3 paper (1 hr at 1800 v, in 0.5 M acetate buffer [pH 4.1]). This allowed separation of cysteic, aspartic, and glutamic acids, neutral amino acids (including amides), γ-aminobutyric acid, and a basic fraction. The neutral fraction was then further separated by paper chromatography, using Whatman 4 paper and a 90:10:29 mixture of 1-butanol-acetic acid-water. Ninhydrin-reacting substances were eluted in 50% ethanol containing 0.01 N HCl, and used for ^{15}N analysis.

^{15}N Experiment. At the beginning of the light period, plants were placed with their roots in a nutrient solution containing 4 mM ^{15}N-nitrate (95 atom %). After 6 hr, the plants were replaced in ^{14}N-nitrate solution until the beginning of the dark period, when this regime was repeated, with 6 hr in ^{15}N, 6 hr in ^{14}N. At intervals, plants were sampled, collecting and freezing the young leaves from three plants for each sample.

^{15}N Analysis. Free ammonia (from total extracts) and amide nitrogen from separated amides were collected by a modification of the distillation procedure of Varner *et al.* (26). The apparatus was reduced in size to handle small samples. Adjustments of pH were carried out with 2 N NaOH instead of borate buffers. Amino acids eluted from paper were treated with 6 N HCl, and samples containing 20 to 50 μg N were dried down in break-seal tubes of about 2 ml volume (8, modified). The Dumas principle was used for sample digestion (5). CaO and CuO were activated and dried at 600 C (6 hr) and a mixture was added to the tubes so as not to mix with the sample during evacuation (10^{-4} mm Hg) and sealing. After digestion, $^{15}N/^{14}N$ ratios were calculated after analysis using a Varian EM600 mass spectrometer, modified for small volume samples.

RESULTS AND DISCUSSION

Young Leaf System. Expanding leaves of pea seedlings show a series of morphologically distinct and recognizable stages; for each leaf, unfolding of leaflets is followed by unfolding of stipules and emergence of the next (folded) young leaf. Under standard growth conditions, these stages can be correlated with changes in various components of the leaf, and with development of other leaves. In this work plants were used at the "4 1/2 leaf stage," when the fourth leaf was fully opened, and the leaflets of the fifth leaf were just beginning to unfold. At this stage, the fifth leaf had approximately half of its final attainable values for dry weight and nitrogen content, while fresh weight and Chl content were about 35% of their final attainable values. At this time, the fourth leaf had already reached maximum dry weight and nitrogen content, but fresh weight was not quite at maximum. During this period of growth, the interval between similar stages of successive leaves was about 4 days. The plants were well established as independent plants, no longer dependent on cotyledonary reserves, but not yet complicated by flowering or extensive senescence (Table I).

Table I. Fresh and dry weights and nitrogen content of various parts of young pea plants at the "4 1/2 leaf" stage, 20 days after germination

Average values per plant were from 8 determinations.

	Fresh wt	Dry wt	Total N	Soluble amino N
	g	mg		μgN
Young leaves (lvs 4+5 + apex)	0.67	67	3.38	218
Growing leaves[1] (lf 5 + apex)	(0.25)	(31)	(2.46)	(115)
Rest of shoot (lvs 1-3)	0.93	64	1.95	156
Roots	1.22	54	2.23	140

[1] As used in later work(1).

Table II. Diurnal changes in levels of total amino nitrogen in young leaves of 20 day old pea plants

The light period began at 0 hr and ended at 12 hr.

Time hr	Amino μmol N/g fresh wt	Time hr	Amino N μmol N/g fresh wt
0(light on)	11.1	12(light off)	11.9
1	13.3	12.5	8.6
2	13.4	13.5	6.8
4	14.0	15	7.3
5	13.2	16	8.0
6	10.1	20	10.0
8	11.1	23	10.8
10	11.8		

Table III. Composition of major free amino acids in young leaves of pea plants, during light (after 10 hr) and dark (after 6 hr) periods.

	nmoles/g Fresh Wt.	
	Light	Dark
Alanine	1120	780
Asparagine	2510	1330
Aspartic acid	1420	2150
Arginine	270	170
Glutamine	1710	1000
Glutamic acid	780	370
Glycine	300	260
Histidine	60	40
Homoserine	3010	2480
Isoleucine	60	140
Leucine	320	220
Lysine	110	120
Methionine	40	40
Phenylalanine	40	40
Serine	270	80
Threonine	1080	600
Valine	100	(trace)
α-aminobutyric	220	430
γ-aminobutyric	550[1]	250[1]
Unknown 'X3'	3000[1]	2500[1]

[1] Approximate values: authentic not available for standardization

Diurnal Changes. Although selected experimental plants were reasonably uniform, the young leaves cannot be regarded as being in a "steady-state" condition. Increase in size and morphological changes are superimposed on diurnal changes in nitrogenous components of the leaves. The light period has at least two effects on nitrogen metabolism. First, light-mediated stomatal opening results in increased transpiration, and a consequent increase in supply of nitrate and organic nitrogen from the root. Second, light supplies energy (and possibly a regulating mechanism) for nitrate reduction and assimilation of ammonia into amino acids. Nitrite reductase (18) and possibly nitrate reductase (11) are dependent on light-generated reducing power. If primary assimilation occurs through glutamine synthetase, which is located in chloroplasts (9, 19), it would be dependent on photophosphorylation, and redistribution of nitrogen through glutamate synthase would require reduced ferredoxin (13).

Diurnal changes in levels of total amino nitrogen are shown in Table II. An increase is seen at the beginning of the light period, at the time when transpiration would increase, followed by a more stable phase after a few hr. In darkness, a transient decrease in levels of amino nitrogen were followed by a gradual recovery. Levels of ammonia also showed small but similar transient changes (Fig. 1). Nitrate levels were also measured but showed no consistent pattern in replications of the experiment. It was found that much of the nitrate was stored, a major part of it in the stem-petiole part of the tissue, and total measurements did not reflect the changes in available nitrate in the leaf. Table III shows a comparison of amino acid composition of young leaves during the stable phases of light and dark periods. For

most amino acids, pool sizes were greater in the light, particularly for asparagine, glutamine, homoserine, glutamic acid, threonine, alanine, and γ-aminobutyric acid. In contrast, α-aminobutyric and particularly aspartic acid showed an increase in the dark. Asparagine, homoserine, and aspartic acid make up over 40% of the soluble pool. An unknown "X3" was present in considerable amounts. This compound emerged from the analyzer just after aspartic acid, and was not any of the following: cysteic acid, methionine sulfoxide, sulfone or sulfoximine, glutathione. A peak running in this region has been identified as glutamyl-alanine (12) but an authentic sample did not co-chromatograph with X3. α-Ketosuccinamic acid (the product of transamination of asparagine) was present in the young leaves but did not appear on amino acid analyses.

Figures 1 and 2 show diurnal variations of some of the amino acids in the young leaves, in this case measured during the ^{15}N experiment. Asparagine changed most conspicuously in phase with the transient changes in total α-amino N, and alanine and homoserine also showed large increases at this time. It is likely that a considerable amount of the asparagine increase is explained by arrival directly from the root in xylem sap. In addition, asparagine probably arrives by reexport (in the phloem) from mature leaves, which receive nitrogenous components in the transpiration stream, yet show no increase in nitrogen content (Urquhart, unpublished).

Analysis of xylem sap gave an indication of the relative composition of nitrogenous compounds transferred from root to shoot, although it was not certain that concentrations in the exuded sap were representative of actual concentrations in the transpiration stream in the intact plant. Amides and amino acids were always present in the sap, but the amount relative to nitrate varied with conditions. The proportion of organic nitrogen was higher with low concentrations of nitrate in the nutrient solution, and also increased with the age of the plant. In plants less than 18 days old, amino acids made up only 15 to 20% of the nitrogen in sap, 20 to 40% at the 4½ leaf stage, and 60% or more in older plants. Asparagine, glutamine, aspartic acid, and homoserine were the main organic components found in the xylem sap. Uptake of nutrient solution (which approximates the delivery of sap to the leaves) was considerably greater (about 2.7-fold) in the light period, and the young leaves accounted for about half of the transpirational flow.

^{15}N Incorporation. ^{15}N-labeled nitrate was used in a preliminary study of the nitrogen metabolism of the young leaves in relation to the rest of the plant. Cycles of both incorporation of ^{15}N and elimination of the label (by ^{14}N) in a number of amino

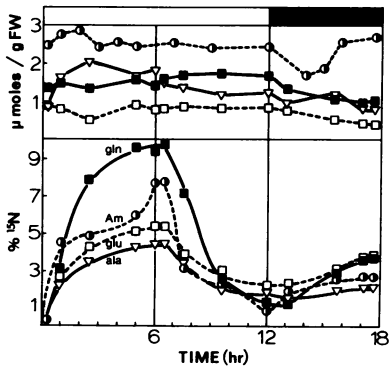

FIG. 1. Pool sizes and levels of ^{15}N in ammonia, glutamine, glutamic acid, and alanine of young pea leaves. ^{15}N-nitrate was supplied to roots of whole plants during hrs 0 to 6 of the light period and 12 to 18 of the dark period; ^{14}N-nitrate was supplied during 6 to 12 of light period. Glutamine data were for the amide group. For ammonia (Am), the pool sizes were not determined in the ^{15}N experiment, and figures shown are from a replicate batch of plants grown under similar conditions.

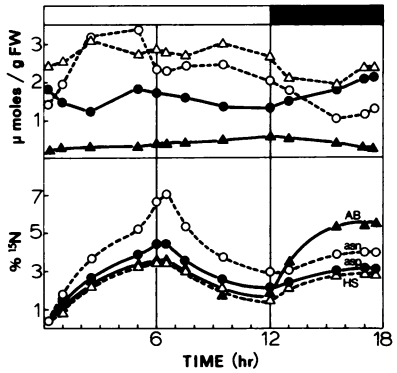

FIG. 2. Pool sizes and levels of ^{15}N in asparagine, aspartic acid, homoserine, and γ-aminobutyric acid of young pea leaves. Whole plants were supplied with ^{15}N-nitrate as described in Figure 1. Asparagine data for the amide group.

acid pools were determined. Experiments were completed both with intact plants (with labeled nutrient solution supplied normally to roots) and with detached shoots (when nutrient solutions were supplied to cut ends of whole shoots cut from the roots at the epicotyl region). In general, the results showed similar trends in both experiments. The data presented here are for whole plants, representing plants under most nearly normal conditions.

Figures 1 and 2 show, for the young leaf portion of the plant, the ^{15}N-labeling data for free ammonia and the most rapidly labeled amino acids and amides, together with pool sizes measured in this experimental material. During the initial labeling period in the light, all compounds measured showed an incorporation of ^{15}N, although the forms of the curves varied. The rate of labeling was rapid at first, and then tended toward a first saturation level after about 3 hr, at values ranging from 3 to 10 atom %. The various saturation levels (which represent "specific activity" of the compound and not total ^{15}N incorporation) and comparison with the value for supplied nitrate (95 atom %) confirmed, as might be expected, the existence of more than one pool for many of the amino acids. Similar results have been described, for example for *Lemna* (2) and *Candida* (27). During the elimination phase in the light, ^{15}N levels fell in all compounds measured. There was little change in size of amino acid pools during this phase, thus continued synthesis and turnover of these pools must occur during the stable part of the light period. Amino acids would be utilized in synthesis of proteins and other nitrogenous materials. The elimination phase might be expected to be complicated by the continuing arrival of ^{15}N-labeled material from root metabolism, while ^{14}N-nitrate-supplied pathways in the leaf would start to operate. This may account in part for the observation that the elimination phase for some amino acids was not so rapid as the original incorporation phase.

Free ammonia (Fig. 1) was the most rapidly labeled compound, and since ammonia is not found in xylem sap this must have been derived from immediate reduction of nitrate arriving in the leaf through the xylem. (Similar results in the experiment using detached shoots confirmed that the leaves were the site of reduction.) The low level for the first saturation shows that either only a small fraction of the ammonia was involved in the active pool, or there was considerable dilution of ^{15}N-nitrate by stored nitrate already in the plant. There are indications that stored nitrate may often be rather inaccessible, for example for nitrate reductase induction (10). At the end of the incorporation period, there was a secondary rise in labeling of ammonia, indicating some change in steady-state conditions. This secondary rise was not observed in the experiment with detached shoots, perhaps indicating a root effect (such as release of ammonia from transported asparagine). On transfer to ^{14}N, the

elimination of label from ammonia was also the most rapid change observed, indicating the continued high rate of turnover. During the ^{15}N incorporation phase in the dark, the formation of ammonia was much slower than in the light. Ammonia labeling did increase slightly, and could be due to light-independent nitrate reduction, or ammonia could arise from breakdown of other components, such as asparagine, which had been labeled in the light period.

In the amino acids, the amide group of glutamine (Fig. 1) showed the most rapid and extensive incorporation of ^{15}N and also showed rapid turnover during the elimination phase. Since glutamine achieved a greater concentration of ^{15}N than the ammonia, it must have been derived from a pool of ammonia with a level of ^{15}N higher than the value measured for the total ammonia pool. Glutamine is a key nitrogen donor for a number of compounds, and is now thought to redistribute nitrogen from the amide group to amino acids through the glutamate synthase system (17). Glutamic acid and alanine were also labeled quite rapidly. In this experiment, it was not possible to determine what proportion of the label in glutamic acid was derived by transfer from glutamine through glutamate synthase, and how much by primary assimilation of ammonia through glutamate dehydrogenase. However, in the accompanying paper (1), it is shown that considerable transfer from glutamine to glutamate occurs in the young leaves. Alanine may be synthesized by primary incorporation of ammonia, or may result from transamination. If transamination is involved, it must occur quickly and from a rapidly labeled primary assimilation product.

The pool of asparagine (Fig. 2) more than doubles during the first 3 hr of the light period, yet label entered the amide group of asparagine more slowly than that of glutamine, suggesting that this is not a primary assimilation pathway in the leaf. The inflexion in the asparagine-labeling curve at the end of the first labeling period (also seen in less pronounced form in the detached shoot experiment) may be due to arrival of asparagine synthesized in the root or in mature leaves. During the elimination phase, the asparagine pool size did not change greatly yet label was eliminated quite rapidly, indicating a considerable turnover of this compound. Aspartic acid, homoserine, and γ-aminobutyric acid were labeled less quickly, and do not seem to be important in primary assimilation of ammonia. It is interesting to note that the two nonprotein amino acids, homoserine and γ-aminobutyric, both showed evidence of substantial turnover in the young leaves.

In the dark, some label was incorporated into all compounds measured, indicating a slow but continuing synthesis of amino acids. Incorporation of ^{15}N into γ-aminobutyric was more rapid in the dark than in the light. This could result from an accelerated decarboxylation of an active pool of glutamic acid. It is also possible that other pathways for synthesis of γ-aminobutyric are operating such as transamination (25). In the final elimination period in the dark (18–24 hr), all pools showed a slight decrease in labeling, and the results have not been shown here.

Similar labeling experiments with detached shoots produced essentially the same results. In general, the labeling was more rapid and more extensive (by a few atom %). This would be expected since label would reach leaves more rapidly, and there would be no interference from stored pools of material from the roots. These observations confirm the belief that the initial labeling patterns in the whole plant result from utilization of nitrate which is transported to the leaf and there reduced and assimilated, and that effects from materials metabolized in the root showed only as a minor factor in later parts of the experiment.

CONCLUSIONS

The pea seedling material described provides a satisfactory experimental system for investigation of amino acid metabolism of young leaves, although diurnal changes in pool sizes and metabolism must be taken into account in the interpretation of any results. The more stable phases in the later parts of the light and dark periods may be more suitable for some types of investigation. The diurnal changes can be correlated with the effects of light on the supply and metabolism of amino compounds.

Under the conditions described here, a large proportion of nitrogen reaches the shoot as nitrate which is reduced and assimilated in the leaf. It is clear that this is predominantly a light-dependent process. The rapid labeling and turnover of a large active pool of glutamine confirm the suggestions, based on presence of enzymes, that in leaves a major proportion of primary assimilation takes place through the amide group of glutamine. A rapid transfer of label from ^{15}N-nitrate to glutamine has also been observed in rice roots (30).

High concentrations of asparagine and homoserine have been noted in peas by other workers (12), but we have been able to show that these compounds not only accumulate but also undergo considerable turnover, as would be required if they are to play an important role in storage and transport. Relatively little is known about the metabolism of asparagine (14). The results here are consistent with enzymic evidence which shows that asparagine may be formed by transfer of the amide group of glutamine, rather than by direct incorporation of ammonia. This is confirmed in the accompanying paper (1) where the metabolism of asparagine and homoserine will be discussed in more detail.

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