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THE METABOLISM OF ASPARAGINE IN PLANTS

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Key Word Index—Angiospermae; asparaginase; asparagine-aminotransferase; asparagine synthetase; diurnal variation; photorespiration.

Abstract—Asparagine is a nitrogen transport compound found in many plants. It is synthesized in seeds by asparagine synthetase upon germination, when protein reserves are hydrolysed, or in roots during nitrate assimilation and dinitrogen fixation. Asparagine synthetase has not been isolated from other plant organs, although evidence suggests that asparagine is synthesized in leaves. Asparagine is catabolized by two separate pathways, deamidation and transamination, releasing nitrogen for amino acid and protein synthesis. Asparaginase activity in many plant species is dependent on potassium and is active in tissues requiring nitrogen for growth such as developing seeds, roots, and leaves. In growing leaves, asparaginase activity undergoes diurnal variation in activity; modification of asparaginase activity has also been observed in microorganisms. Asparagine aminotransferase activity is present in pods and developing and mature leaves, possibly supplying nitrogen for leaf growth through the photorespiratory pathway, and is not found in other organs. In older leaves nitrogen is not required for growth, and several reports have suggested that transpirationally derived asparagine in older leaves undergoes little metabolism and is re-exported to the apex, or to developing fruits.

INTRODUCTION

Actively growing roots, leaves and seeds require large quantities of reduced nitrogen for the synthesis of amino acids, proteins, etc. To meet this demand, nitrogen acquired by the roots is transported in organic form in the xylem and phloem, or as nitrate in the xylem only. A restricted group of organic compounds are used for nitrogen transport, and usually have low carbon to nitrogen ratios, high solubility, stability and mobility in physiological fluids (for recent reviews see [1–3]). Asparagine meets these criteria and is thus well suited to its role as a transport compound that is widely distributed in plants. At the time that Chibnall [4] wrote his review of protein metabolism in plants (1939), the role of asparagine in plants as a nitrogen transport compound, as a detoxification product, and as a compound that varied in concentration in the light and dark was known, yet the mechanisms accounting for the synthesis or degradation of asparagine were still uncertain.

The metabolism of asparagine was reviewed in 1975 [5], and in the early 1980's [1, 6], but many new aspects pertaining to the synthesis and degradation of asparagine in higher plants have recently been reported and are the subject of this review.

ASPARAGINE AS A TRANSPORT COMPOUND

Since photosynthetically derived carbon skeletons must be first transported to the root for the synthesis of organically combined nitrogen compounds, the low C:N ratio of asparagine (2:1) suggests carbon conservation [7]. Asparagine is more soluble than the ureides [3], is more stable than glutamine [8] or the ureides in solution [9] and it is mobile at physiological pH [2, 10]. The amides asparagine, and, to a lesser extent, glutamine, are the main nitrogen constituents of the xylem sap of many plants including nodulated or non-nodulated temperate legumes (e.g. *Pisum*, *Lupinus*, *Vicia*, *Trifolium*, *Medicago*, and *Lotus* [3, 11]), non-nodulated tropical legumes (e.g. *Glycine*, *Vigna*, *Phaseolus* [3, 7]), several actinorhizal plants (e.g. *Casuarina*, and *Myrica* [3]), and others, while the ureides are important nitrogen transport compounds found in nodulated tropical legumes (e.g. *Glycine*, *Vigna*, *Phaseolus* [3, 7]). Recently, asparagine has been reported to be a major component of the xylem sap in mature nodulated or nonnodulated peanuts (*Arachis*), with 4-methyleneglutamine being important in younger plants [12].

The amides are important products of protein degradation, synthesized upon hydrolysis of storage proteins during seed germination [13–15], or the breakdown of proteins during leaf senescence, and are subsequently transported to developing leaves or fruits through the phloem [16]. The amides (along with other key amino acids) may also be synthesized during exposure of plants to periods of environmental stress such as mineral deficiencies, drought, or conditions of increased salinity [17].

In developing pea leaves and seeds, asparagine has been found to be actively metabolized [18, 19], but in mature leaves that no longer require nitrogen for growth, asparagine is not readily metabolized and is re-exported (in the phloem) from the leaf to regions of active growth such as developing leaves and seeds [10, 20]. Asparagine is the

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major nitrogenous compound detected in the phloem of several legumes and concentrations up to 30 mM have been reported [18]. The process of re-export of asparagine from this mature leaf to the phloem involves xylem to phloem transfer with little metabolism of asparagine in the mature leaf [2, 9, 21]. The apparent limited involvement of asparagine in cellular processes in mature plant organs has lead to much confusion regarding its role in plants since it was first isolated, and two reports have even concluded that asparagine was a 'dead-end' metabolite [22, 23].

SYNTHESIS OF ASPARAGINE

Asparagine was first observed in *Asparagus sativus* by Vauquelin and Robiquet in 1806; and thereafter a relationship between light and asparagine became apparent when, in 1858, Sullivan noted that etiolated vetch seedlings accumulated high levels of asparagine, which decreased rapidly when the seedlings were exposed to light [4]. The relationship between the increase of asparagine in the dark and its decrease in the light has been reported many times [e.g. 5, 16, 24–26] but the significance of this has remained unclear. Prianischnikow, in 1922 [4], first observed that asparagine concentrations increased in light in the presence of ammonia and suggested that the accumulation of asparagine may be a mechanism of ammonia detoxification, an observation recently confirmed (see below) [27]. The role of asparagine as a detoxification product, acting as a nitrogen storage compound synthesized under high ammonia conditions, has now been firmly established [28].

Three routes have been proposed to account for asparagine synthesis (Fig. 1). One involves the synthesis of asparagine from 2-oxosuccinamic acid (the β-amide of oxaloacetic acid), and a suitable amino donor in a reverse reaction of asparagine:oxoacid transaminase (EC.2.6.1.14) (Fig. 1a). This reaction has been detected in rat liver extracts, the preferred amino donors for asparagine synthesis being α-aminobutyric acid, alanine, glutamine, and norvaline, although several other amino acids participated in the reaction [29], but, has been demonstrated only at low levels in leaves of *Pisum sativum* (20-fold less activity than the asparagine-utilizing direction of the reaction, [30]) and is of questionable physiological importance in plants.

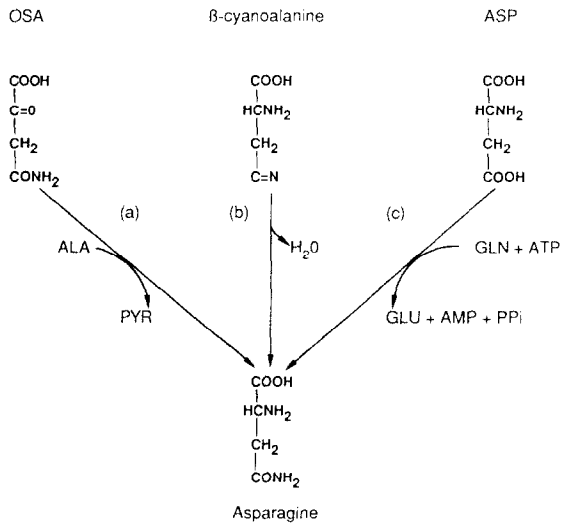


Fig. 1. Enzymes responsible for the synthesis of asparagine in plants. (a) Asparagine-oxoacid transaminase, (b) β-cyanoalanine synthase and (c) asparagine synthetase. Oxosuccinamic acid: OSA.

A second pathway of asparagine synthesis involves the incorporation of cyanide and cysteine into β-cyanoalanine (catalysed by β-cyanoalanine synthase, EC. 4.4.1.9) which is then hydrolysed (β-cyanonalanine hydrolase, EC. 4.2.1.65), producing asparagine (Fig. 1b). This pathway of asparagine synthesis has been demonstrated in a variety of plants. For example, both enzymes have been isolated in *Lupinus angustifolius* seedlings and cotyledons [31–33] and *Asparagus officinalis* seedlings [23], and β-cyanoalanine synthase has been detected in a wide range of plants [33]. Active β-cyanoalanine synthase (10-fold higher activity than asparagine synthetase activity) has also been demonstrated in root tips of *Zea mays* [34]. The major source of cyanide in plants, however, is thought to be cyanogenic glycosides which only occur in a limited range of plants [35, 36], and therefore the role of the β-cyanoalanine pathway as a sole route of asparagine synthesis was questioned [1]. This pathway is generally considered to be of importance only as a cyanide-detoxifying mechanism [1], but this has recently been challenged [37]. Another possible source of cyanide in plants was proposed by Solomonson and Spehar [38] in a study on the regulation of nitrate reductase activity in *Chlorella vulgaris*. They observed that hydroxylamine, an intermediate of nitrite reduction, and glycolate, an intermediate of the photorespiratory pathway, produced cyanide in the presence of a soluble extract from *Chlorella*. This reaction has also been confirmed in leaves of spinach, maize, and barley, and is dependent upon ADP and Mn^{2+} for optimal activity [39]. Recently, cyanide synthesis has been observed during ethylene biosynthesis [37, 40, 41], again rekindling the debate regarding the importance of the β-cyanoalanine pathway in asparagine synthesis.

The third route leading to asparagine synthesis is mediated by asparagine synthetase (EC. 6.3.5.4) (Fig. 1c), and involves an ATP-dependent transfer of the amide group of glutamine (or in some cases ammonia) to aspartate (in the presence of Mg^{2+}) producing asparagine, glutamate, AMP and PPi [42]. Asparagine synthetases from all plant sources examined so far are very unstable, and require the addition of protectants (DTT, mercaptoethanol, and glycerol) in order to detect activity. Other confounding factors that affected detection of asparagine synthetase activity in crude preparations include the presence of inhibitors [25, 43, 44], asparaginase activity (especially in nodule preparations, e.g. [45]), glutaminase activity [46], and in the case of ammonia as substrate, glutamine synthetase activity [1].

The first report of asparagine synthesis in plants [47] indicated that ammonia or hydroxylamine were incorporated into asparagine in an ATP-dependent reaction analogous to glutamine synthetase. This conclusion was consistent with feeding experiments demonstrating that asparagine concentrations increased in leaf discs infiltrated with aspartate, ammonia and ATP [e.g. 48]. However, recent feeding experiments, using leaf discs from soybean, have supported the role of glutamine as nitrogen donor for asparagine synthesis [27]. Infiltration of the leaf discs with aspartate and ammonia enhanced the synthesis

of asparagine, consistent with earlier reports [48], but the increase in asparagine levels was reduced in the presence of methionine sulfoximine (MSO), an inhibitor of glutamine synthetase. When glutamine was supplied to the leaf discs, an enhancement of asparagine levels was also noted and this increase was not affected by MSO suggesting that glutamine was the direct nitrogen donor for asparagine synthesis [27]. A rapid conversion of [^{14}C]asparatate to [^{14}C]asparagine (as well as organic acids) has also been observed in nodules of *Medicago* [11]. Active GS (and glutamate synthase, GOGAT) activity were suggested to be required for asparagine synthesis, since the appearance of [^{14}C]asparagine in xylem exudates decreased by over 80% in the presence of MSO and azaserine (an inhibitor of GOGAT activity) [11]. Enzymological studies have also indicated that glutamine, and not ammonia, is the preferred nitrogen donor for asparagine synthetase extracted from germinating cotyledons (*Glycine max*, [36]; *L. luteus*, [42, 46]; and *L. albus*, [49]; *Gossypium hirustum*, [13]), and root nodules (*L. angustifolius*, [50]; *G. max*, [45]). Stulen *et al.* [34] reported that asparagine synthetase extracted from mature *Z. mays* roots displayed only a marginal preference for glutamine over ammonia. From this and other observations Oaks and Ross [51] suggested that asparagine synthetase from maize root may be different from legume cotyledon and nodule enzymes.

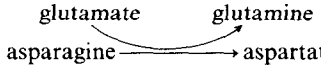
The occurrence of asparagine synthetase activity in different plant organs is not well documented. The absence of asparagine synthetase in *Asparagus officinalis* seedlings (isolated without added protectants) led Cooney *et al.* [23] to conclude that the β-cyanoalanine pathway was responsible for asparagine synthesis. Asparagine synthetase activity was also absent from leaves of *P. sativum* (extracted with various protectants, [25]), even though detached shoots were able to synthesize asparagine from aspartate. Bauer *et al.* [52] demonstrated an increase and decrease of ^{15}N in asparagine in detached pea leaves fed $^{15}NO_3^-$, followed by $^{14}NO_3^-$, which indicated that asparagine was synthesized and turning over in leaf tissues; furthermore, aspartate-dependent asparagine synthesis was also demonstrated in soybean leaf-disc feeding experiments [27].

Several reports, from plant and animal sources, have indicated that asparagine synthetase is sensitive to several cations and anions. For example, asparagine synthetase, and associated glutaminase activities were observed to be stimulated by Cl^- in mouse leukemia cells [53] and *L. luteus* cotyledon extracts [46], and a strong inhibition of lupin cotyledon asparagine synthetase has been reported in the presence of Ca^{2+} [42, 44].

A heat-stable, dialysable inhibitor (possibly Ca^{2+}, [44]) of lupin-cotyledon asparagine synthetase was found in pea leaf homogenates [25], and these authors cautioned against dismissing asparagine synthetase as a plausable route for asparagine synthesis just because activity is not detected in extracts. Homogenates of asparagus seedlings, in which asparagine synthetase could not be detected [23], were observed to strongly inhibit asparagine synthetase activity obtained from lupin cotyledons [25], suggesting that detection of asparagine synthetase activity in leaf or other tissues may be impeded by inhibitors complexing with the enzyme during extraction. The presence of a heat-labile, nondialysable inhibitor of asparagine synthetase (not present in 3-day-old cotyledons), was also observed in developing *Vigna radiata* seedlings, accounting for reduced asparagine synthetase activity noted in older seedlings [43].

BREAKDOWN AND UTILIZATION OF ASPARAGINE

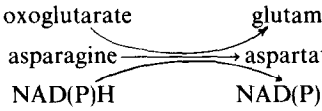
Asparagine has been proposed to be catabolized by four routes, although the first two of these have been questioned in the literature since the enzymes responsible for the reactions have not been isolated. The first of these routes involves the transfer of the amide group of asparagine to glutamate forming glutamine through the reversal of asparagine synthetase (reaction 1) as concluded by Baurerova and Shorm in 1959 in a study on rape seedlings [see 54].



(1)

This route of asparagine metabolism was also supported by ^{15}N experiments in *P. sativum* leaves, demonstrating a rapid incorporation of ^{15}N, from [^{15}N]amide asparagine into glutamine [55], but this was not observed by Atkins *et al.* [56] in *Lupinus*. It is also possible that the labelling of glutamine in the above studies may be a result of asparagine deamidation followed by the assimilation of the ammonia into glutamine by glutamine synthetase, but this has not been tested.

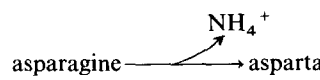
A second suggested route involves the reductive transamidation of asparagine with 2-oxoglutarate (reaction 2), in an analogous manner to glutamate synthase detected by the asparagine-dependent oxidation of NAD(P)H [57, 58].



(2)

This route has been questioned since the asparagine used was probably contaminated with aspartate, which, in the presence of an oxo-acid would be transaminated, and the oxaloacetic acid produced would then be reduced by malate dehydrogenase thereby consuming NAD(P)H [59].

The third and fourth routes of asparagine metabolism have been confirmed and their importance established by identification of the presence of the appropriate enzyme, by feeding studies, and the use of metabolic inhibitors. One involves the hydrolysis of asparagine, catalyzed by asparaginase (E.C. 3.5.1.1.) to produce aspartate and ammonia (reaction 3), and the other, the transamination of asparagine (in the presence of an oxo-acid) producing 2-oxosuccinamic acid and an amino acid (reaction 4).



(3)

(4)

These two reactions appear to be the principal routes by which asparagine is metabolized in higher plants.

DEAMIDATION AND TRANSAMINATION OF ASPARAGINE

In plants, the hydrolysis of asparagine by asparaginase, producing aspartate and ammonia, was initially detected

in roots of germinating barley seeds by Chibnall and Grover in 1926 [4], although the activity of this preparation was quite low [60, 61]. In 1946 Greenstein and Carter [62] noted weak deamidation of asparagine (detected by ammonia production) in aqueous rat liver extracts which increased when pyruvate was added to assay mixtures. These results were interpreted to involve two separate reactions: the condensation of asparagine and pyruvate to form a dehydropeptide followed by the cleavage of dehydropeptide to form pyruvate and ammonia. Further work demonstrated that two 'asparaginases' were present; one was heat-labile (asparaginase I) and unaffected by pyruvate, while the other (asparaginase II), was heat-stable and activated by pyruvate [63, 64]. Several years later, Meister and co-workers, again using rat liver extracts, demonstrated that the pyruvate-stimulated asparagine deamidation was due to the combined action of asparagine transamination, producing 2-oxosuccinamic acid, which was then deamidated by ω-amidase, to yield oxaloacetic acid and ammonia [29, 65–69]. Meister [67] also demonstrated that spinach and lettuce leaves contained ω-amidase activities, capable of hydrolysing oxosuccinamic acid (and oxoglutaramic acid, the β-amide of oxoglutarate), and suggested that the associated transaminase activity might be present.

Asparagine transaminase was initially detected in plants by Wilson *et al.* [70], Yamamoto [48], and later by Gamborg [71] and Cincerova [72]. Yamamoto [48] and Cincerova [72] reported that asparagine transamination occurred in the presence of oxoglutarate, an observation which has not been supported [30]. It is likely that the asparagine solutions used in their assays contained aspartate (a common contaminant present in asparagine) which would readily transaminate in the presence of oxoglutarate and other oxo-acids [73].

In 1954 Kretovitch and co-workers [see 60] obtained extracts from lupin, pumpkin, and corn which contained amidases capable of splitting asparagine and glutamine to form ammonia. At about this time, Webster and Varner [47] reported on the synthesis of asparagine from aspartate and ammonia. However, work a decade later was unable to confirm either the synthesis, or the hydrolysis of asparagine in wheat [22] or cotton seedlings [35] and cast doubt upon the role and metabolism of asparagine in plants.

Several years after the observation that asparagine hydrolysis could not be detected in young seedlings, Lees and Blakeney [61] reported on the presence of asparaginase in extracts obtained from mature *Lupinus luteus* (in K-Pi buffer). High asparaginase activity was found in both the plant and bacteroid fractions of nodules and in root extracts, while only low levels of activity occurred in the leaves (age unspecified). They also pointed out that roots contained little free amide while the bulk of the asparagine was found in the leaves, and they suggested that this was consistent with the data on the distribution of asparaginase [61]. Streeter [74] concluded that asparaginase activity in the plant fraction of nodules, prepared from 30-to 70-day-old *G. max*, was probably a contaminant derived from bacteroid-asparaginase during extract preparation. However, Scott *et al.* [50], analysing ammonia assimilation in nodulated *L. angustifolius*, also detected asparaginase in the plant fraction of nodules (extracted in K-Pi buffer), and noted that the activity decreased during the first two weeks after nodulation. They postulated that the asparaginase detected in the plant fraction of the nodule hydrolysed incoming asparagine, synthesized from cotyledonary reserves, and thereby supplied nitrogen and carbon required for nodule growth prior to the establishment of active nitrogen fixation (suggesting that asparaginase obtained from the plant fraction was different from nodule asparaginase).

Lea and Fowden [5] criticised the work of Lees *et al.* [22] and Ting and Zchoche [35] on asparagine metabolism in seedlings and suggested that a more likely site of asparagine metabolism would be one where nitrogen is required for growth, such as developing seeds involved in active protein synthesis. For example, Lea *et al.* [75] demonstrated that *P. sativum* cotyledons, maintained in sterile culture with asparagine as the sole nitrogen source, could support high rates of protein synthesis, and a more recent report by Skokut *et al.* [76] suggested that asparagine was deamidated in cultured cotyledons of *G. max* fed with [^{15}N]amide asparagine. However, many studies failed to demonstrate appreciable levels of asparaginase activity in developing seeds from a range of plants, such as *Pisum*, *Phaseolus*, *Vicia* [77], *Pisum* [78], several *Lupinus* varieties [80, 79], or in *Zea* endosperm tissue [81].

The intermittent occurrence of asparaginase activity in plants was partially explained in a report by Sodek *et al.* [82], who demonstrated that asparaginase, obtained from developing seeds of *P. sativum*, was dependent on the presence of potassium for activity. Maximum asparaginase activity in crude extracts obtained in Tris-HCl buffer was obtained when 20–50 mM potassium chloride was added to the assay solution. Developing seeds from *P. arvense*, *Phaseolus multiflorus*, *Vicia faba*, *L. albus*, *L. mutabilus*, *Z. mays*, and *Hordeum vulgare* all demonstrated stimulation of their asparaginase activities on the addition of potassium. Chang and Farnden [83] reported the occurrence of two potassium-independent asparaginases in developing seeds of *L. arboreus* and *L. angustifolius*, although preparations from roots, leaves, developing nodules, and flower buds required potassium for activity. In another study [79], 54 *Lupinus* varieties (including several species) were challenged with antiserum raised against a potassium-independent asparaginase from *L. polyphyllus*. Eleven lines were found to have potassium-independent asparaginases, while other varieties had immunologically distinct potassium-dependent asparaginases [79]. Asparaginase obtained from a *Chlamydomonas* species was also found to be potassium-independent [84, 85].

Prior to the observation of the potassium dependency of asparaginase, utilization of asparagine in developing seeds was understood in several species, although the sporadic occurrence of the enzyme was indeed baffling. In a comprehensive study on nitrogen utilization during seed development, Atkins *et al.* [18] demonstrated that asparagine was metabolized in developing *L. albus* seeds. They fed uniformly labelled [^{14}C], [^{15}N]amide asparagine via the transpiration stream to fruiting shoots and noted that the bulk of the carbon and nitrogen label was translocated to the seeds as asparagine. Most of the ^{15}N in the endosperm fluid was recovered in ammonia, glutamine, and alanine, while most of the ^{14}C was found in non-amino compounds. As the seeds developed, both ^{15}N and ^{14}C were detected in amino acid constituents of seed storage proteins. Asparaginase activity, assayed in crude

embryo extracts, increased *ca* 10-fold during seed development and reached maximum activity six to seven weeks after anthesis (extracts were obtained in phosphate buffer, cation unspecified, although Lea *et al.* [79] indicated that the cation was potassium). Asparaginase was present in trace amounts in the seed coat, and liquid endosperm, but the bulk of activity was present in the embryo. The increase in asparaginase activity during seed development also preceded the increase in embryo fresh weight, supporting the role of asparagine as a nitrogen transport compound, supplying nitrogen required for growth [18]. Similar observations were reported for changes in asparaginase activity in developing seeds from two varieties of *P. sativum* (extracted in potassium phosphate buffer [86]). An increase in the protein levels in the cotyledon followed the rise in asparaginase activity by several days (for related enzymes of nitrogen metabolism in developing seeds see [78, 87, 88]).

In 1977 Streeter [74] detected active transamination of asparagine in leaves of *G. max* while only marginal asparaginase activity was observed (age unspecified, extracted in Tricine buffer). Based upon the high specific activity of the transaminase (higher than the specific activity of asparaginase), he suggested that transamination was the major metabolic route for asparagine metabolism in leaves. Lloyd and Joy [89] noted that after feeding [^{14}C]asparagine to leaves of *P. sativum* and *G. max*, label appeared in hydroxysuccinamic acid (see Fig. 2). This compound was also formed when 2-oxosuccinamic acid was fed to leaves, and they suggested that following asparagine transamination, 2-oxosuccinamic acid was reduced to hydroxysuccinamic acid.

After the observation that asparaginase activity in many plants was potassium-dependent, other studies again described the intermittent occurrence of asparagine metabolism in plants, noting that asparaginase and asparagine aminotransferase activities were localized spatially and temporally within the plant. Asparaginase activity was found to decrease with leaf age in *P. sativum* [90], *L. albus* [91], and with increasing distance from the root-tip in two lupin species [83]. The absence of asparaginase in mature plant organs has also been confirmed immunologically by Lea *et al.* [79] using antisera prepared against asparaginase of developing *L. polyphyllus* cotyledons.

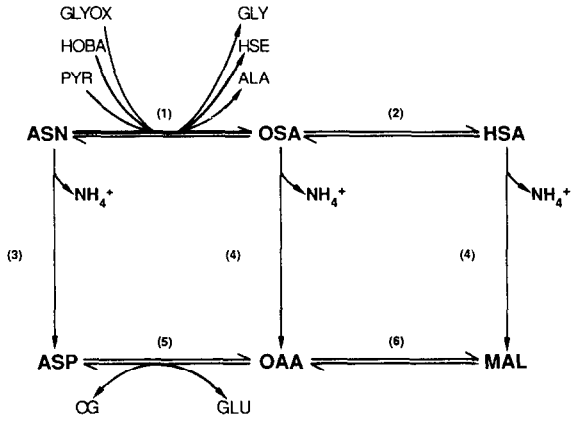


Fig. 2. Pathways of asparagine degradation in leaves of *Pisum sativum* (1) asparagine-oxoacid transaminase, (2) reduction, (3) asparaginase, (4) ω-amidase, (5) aspartate-oxoglutarate transaminase and (6) malate dehydrogenase. Reactions (1) and (5) are inhibited by aminooxyacetate and reactions (3) and (4) by 5-diazo-4-oxo-L-norvaline. Ammonia is assimilated by glutamine synthetase, a reaction inhibited by MSO. Glyoxylate: GLYOX, homoserine: HSE, 4-hydroxy-2-oxobutyric acid: HOBA.

During leaf development asparagine aminotransferase activity remained constant in *P. sativum* [90], but was observed to increase in developing leaves of *L. albus*, and decrease upon senescence [91]. Asparagine aminotransferase and asparaginase activities are present at low levels in pods, and transaminase activity is absent from developing seed and root extracts [86]. Walton and Woolhouse [92] have also reported high levels of asparagine aminotransferase activity in *P. sativum* leaves, while cotyledon and root apices exhibited only trace activities.

CONFIRMATION OF PATHWAYS OF ASPARAGINE CATABOLISM IN LEAVES

Feeding experiments have corroborated the enzymological data, and confirmed that asparaginase is important in supplying nitrogen and carbon during seed maturation in *Lupinus albus* [18], and that asparaginase and asparagine transaminase are the two main routes for asparagine metabolism in *Pisum sativum* leaves; as leaves developed, asparagine metabolism changed primarily from deamidation to transamination [19, 90, 93, 94]. For example, the fate of [^{15}N]amino asparagine, fed via the transpiration stream at three stages of leaf development (young, half expanded and mature leaves), demonstrated that asparaginase activity decreased with leaf age since (1) the proportion of ^{15}N in aspartate, the deamidation product of asparagine, and glutamate, a transamination product of aspartate, decreased with leaf age, (2) the addition 5-diazo-4-oxo-L-norvaline (DONV, an inhibitor of several deamidases, [see 94]) reduced ^{15}N recovery in aspartate and glutamate in young leaves more so than older leaves, and (3) addition of aminooxyacetate (AOA, an inhibitor of transaminases) had no affect on the recovery of ^{15}N in aspartate, yet reduced the presence of ^{15}N in glutamate in all leaf ages (Fig. 2) [93]. It was also noted that asparagine transamination may lead directly to homoserine synthesis since ^{15}N rapidly appeared in homoserine during feeding experiments and the incorporation of ^{15}N in homoserine was AOA sensitive [19]. This pathway of homoserine synthesis (i.e. transamination of 4-hydroxy-2-oxobutyric acid (Fig. 2) with asparagine) [95] is different from the accepted route of synthesis, from aspartate, mediated by aspartate kinase, aspartate semi-aldehyde dehydrogenase, and homoserine dehydrogenase [95, 96].

Other experiments [19] in which [^{15}N]amide asparagine was fed to half expanded leaves indicated that ^{15}N was recovered predominantly in hydroxysuccinamic acid (reduction product of oxosuccinamic acid [89]), and the amide group of glutamine. The recovery of ^{15}N in glutamine was greatly reduced by MSO, while the levels of ^{15}N in ammonia increased, consistent with deamidation and the inhibition of ammonia assimilation by glutamine synthetase. When MSO and AOA were added together the recovery of ^{15}N in hydroxysuccinamic acid and ammonia was reduced, showing that deamidation had occurred after the transamination of asparagine (Fig. 2)

[19, 94]. These data demonstrated that both transamination and deamidation of asparagine play an important role in supplying nitrogen to developing leaves.

It is interesting to note that in developing leaves, the final products of asparagine degradation, OAA and ammonia, are equivalent regardless of their catabolic pathway (deamidation would produce ammonia and aspartate which is readily transaminated to OAA, or transamination yields oxosuccinamic acid which is deamidated to produce OAA and ammonia); however, each route plays an important role during leaf growth. The deamidation of asparagine is favoured early in leaf development since both the nitrogen and carbon of aspartate are required for the synthesis of the aspartate family of amino acids (lysine, methionine, threonine, and isoleucine), while transamination of asparagine would supply nitrogen for the synthesis of glycine and serine, intermediates of the photorespiratory pathway (see below).

ASPARAGINE AND PHOTORESPIRATION

In more detailed studies on the enzymology of asparagine transamination in *P. sativum*, Ireland and Joy [30, 97] suggested that the amino-nitrogen of asparagine could be incorporated into the photorespiratory pathway in developing leaves. This conclusion arose from two observations, the first being that the aminotranferase responsible for metabolizing asparagine co-purified with serine-glyoxylate aminotransferase, an enzyme of the photorespiratory pathway [30]. The second observation was that asparagine aminotransferase activity co-sedimented and co-migrated with catalase activity (a peroxisomal marker) during either differential, or sucrose density gradient centrifugation [97]. These results demonstrated that asparagine aminotransferase was located in the same subcellular compartment, and was in fact the same enzyme, as serine-glyoxylate aminotransferase. It was also concluded, in the latter study, that asparaginase was a cytosolic enzyme [97].

The enzymological data, linking asparagine transamination with photorespiration, was supported by experiments feeding [^{15}N]amino asparagine to developing pea leaves in conjunction with photorespiratory inhibitors [93, 98, 99]. After supplying [^{15}N]amino asparagine through the transpirational stream, label was recovered in serine and glycine, intermediates of the photorespiratory pathway. When glycolate oxidase was inhibited with α-hydroxy-2-pyridine methanesulphonic acid (HPMS, thereby reducing the synthesis of glyoxylate), the recovery of ^{15}N in glycine decreased, while at the same time, label increased in alanine and glutamine. The inhibition of glycine decarboxylase and serine hydroxymethyltransferase, enzymes responsible for the conversion of glycine to serine, with isonicotinyl hydrazide led to an increase of ^{15}N in glycine [99]. The increase or decrease of ^{15}N in these and other amino acids was consistent with the scheme of photorespiration as it is currently thought to proceed [e.g. 100, 101], and suggested that asparagine is involved in supplying nitrogen during photorespiration. The photorespiratory pathway was therefore proposed to be an open ended series of reactions supplying nitrogen for glycine, serine, and glutamate synthesis during the period when the leaf was still acquiring nitrogen for growth [99], rather than a closed cycle [e.g. 100, 102, 103]. Recent work has indicated that asparagine contributes approximately 7% of the nitrogen to glycine during photorespiration in half expanded pea leaves [98].

The metabolic fate of oxosuccinamic acid and hydroxysuccinamic acid, which result from asparagine transamination, are not well understood in plants. Deamidation of oxosuccinamic acid in plants has been suggested in the literature [e.g. 19, 67, 72, 74, 94] even though the enzyme ω-amidase has not been isolated from plants (it has been characterized from rat liver) [104]. However, feeding experiments with [^{14}C]asparagine suggest that a strong equilibrium towards hydroxysuccinamic acid exists [89]. Deamidation of hydroxysuccinamic acid in crude extracts was only observed at low rates possibly indicating that hydroxysuccinamic acid may reconvert to oxosuccinamic acid prior to metabolism. The majority of the label from [^{15}N]amide hydroxysuccinamic acid, fed through the transpirational stream to half expanded pea leaves, was detected in the amide group of glutamine suggesting that deamidation of hydroxysuccinamic, or oxosuccinamic acids, and reassimilation of ammonia by glutamine synthetase, take place *in vivo* [94].

REGULATION OF ASPARAGINE METABOLISM

Little work has been published on the regulation of asparagine synthesis or degradation in plant tissues. Asparagine synthetase in cotyledonary tissues is responsible for the synthesis of asparagine during germination and early seedling growth, and this enzyme appears to be subject to regulation since activity increases during the first two to four days of seedling development and then declines [43, 49]. The increase in this activity during early seedling growth is abolished in the presence of cycloheximide [43]. This enzyme from cotyledonary sources is also inhibited in the presence of the products of the reaction, asparagine [43, 49, 51], AMP, PPi [40, 49], as well as ADP [51], high concentrations of ATP [49] and several cations, especially Ca^{2+} [42, 44]. Rognes [46] noted that Cl^- is effective in activating glutamine-dependent asparagine synthetase activity (Cl^- has no effect on the ammonia-dependent reaction), by enhancing the affinity for glutamine 50-fold. However, associated glutaminase activity also increased (30-fold) in the presence of this anion.

The deamidation of asparagine by asparaginase also appears to be regulated in several plant organs. Asparaginase activity increases in developing cotyledons shortly after flowering [82, 84], decreases during leaf development [90, 91], and varies diurnally in developing pea [26, 105] and soybean leaves (Sieciechowicz, K. A., unpublished results) with a three to five fold increase in activity observed during the light period. Light was demonstrated to be required for the increase in asparaginase activity obtained from half-expanded pea leaves since (1) increased energy-fluence-rates resulted in an enhancement of extractable asparaginase activity [105]; (2) activity in young pea leaves was maintained at high levels during prolonged light period treatments, and present at low levels when plants were exposed to continued dark periods [26, 105]; (3) inhibition of photosynthetic electron transport (with DCMU or atrazine) prevented the increase in asparaginase activity and photosynthetic activity [105]; (4) The increase in activity was not mediated by phytochrome [105]. There were no indications of circadian rhythmicity [26, 105].

An increase in extractable asparaginase activity from a marine *Chlamydomonas* species was also dependent on

changes in energy-fluence-rate, and sensitive to the presence of DCMU [84]. These authors reported that a four-fold increase in activity, observed in the absence of external nitrogen, required 48 hr. Since the increase in activity was sensitive to the addition of cycloheximide, they concluded that *de novo* protein synthesis was involved.

Several studies have indicated that microbial asparaginases are subject to regulation. An internal yeast asparaginase (asparaginase I) responds to increased substrate concentration in a non-Michaelis–Menten manner, and higher levels of activity are not a result of protein synthesis, whereas external yeast asparaginase (asparaginase II) does exhibit Michaelis–Menten kintics, and increased activity does arise from *de novo* protein synthesis, under conditions of low external nitrogen [106, 107]. Jerebzoff–Quintin and Jerebzoff [108] proposed that reversible phosphorylation of a high M_r aggregate, exhibiting asparaginase activity, may account for cyclic asparaginase activity observed during the sporulation rhythm of *Leptosphaeria michotii*.

Post-translational modification of developing pea-leaf asparaginase through phosphorylation/dephosphorylation was not responsible for the variations in activity observed [109]. Extractable asparaginase activity increased two-fold in the presence of Ca^{2+}, but this response was not dependent upon calmodulin. Studies with transcriptional and translational inhibitors suggested that mRNA, and protein synthesis are required for increased activity, and that proteolytic degradation of asparaginase is responsible for the decrease in activity in the dark [109]. We have recently observed that the extent of the diurnal variation in asparaginase activity also increases in successive developing leaves as plants age, indicating that asparaginase activity is not the same in each young or half expanded leaf during plant develpment [110].

Since high levels of ammonia (above 1 mM) are dangerous to photosynthesizing plant cells [28] and result in the shift of metabolism, predominantly from sugar synthesis to increased amino acid synthesis [e.g. 111–115], reactions giving rise to ammonia might be expected to be under some form of regulation, so that ammonia is produced when it can be assimilated and further metabolized most efficiently. Assimilation of ammonia by glutamine synthetase, and the subsequent metabolism of glutamine are dependent on products of the light reactions of photosynthesis [103], and it has been suggested that the principal source of ATP for glutamine synthetase activity is supplied by photophosphorylation [116]. It is not surprising then, to see a light-dependent increase in asparaginase activity which is possibly a strategy to ensure that free ammonia is produced mainly during the light period when assimilation of ammonia and subsequent metabolism of glutamine are optimal. Since high levels of free ammonia in darkness are potentially dangerous to a cell, the decrease in asparaginase activity in the dark is another important aspect of asparagine metabolism.

CONCLUSIONS

Despite asparagine being the first amino acid detected in plants, there has been little definitive work done on its metabolism. Only in the last few years has a reasonably clear picture of its synthesis and degradation emerged. Indeed, it has taken over a hundred years to begin to elucidate the relationship between light and asparagine, first observed by Sullivan and Boussingalt in the mid 1800's [4]. The metabolism of asparagine and glutamine are interwoven, and this has often lead to the asumption that the synthesis and degradation of asparagine take place in much the same manner as glutamine. However, glutamine is readily metabolized in various plant organs, whereas asparagine is metabolized selectively. The value of asparagine as a transport compound is enhanced by the limited capacity for its catabolism in plant tissues, mainly found in organs requiring both nitrogen and carbon for amino acid and protein synthesis.

Many questions remain to be answered regarding the synthesis and degradation of asparagine. It is synthesized in non-nodulated roots and mature leaves, but the route of synthesis is far from clear; asparagine synthetase has not been isolated from leaf tissues where active inhibitors of this enzyme can be present; the significance of hydrolysis of β-cyanoalanine (producing asparagine), and the extent of its occurrence in leaves or roots is not known. Asparagine is degraded through two routes in developing leaves; transamination and deamidation. The subsequent metabolism of oxosuccinamic acid, a product of asparagine transamination, is not fully understood nor have the relevant enzymes been isolated. Asparaginase is turned over rapidly in developing leaf tissues; the regulation of the synthesis and degradation of asparaginase still remain to be elucidated.

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