Plant Physiol. (1983) 73, 165–168
0032-0889/83/73/0165/04/$00.50/0

Asparagine Synthesis in Pea Leaves, and the Occurrence of an Asparagine Synthetase Inhibitor[1]

Received for publication February 14, 1983 and in revised form May 10, 1983

KENNETH W. JOY, ROBERT J. IRELAND, AND PETER J. LEA
Biology Department and Institute of Biochemistry, Carleton University, Ottawa, Ontario K1S 5B6 Canada (K. W. J., R. J. I.); and Biochemistry Department, Rothamsted Experimental Station, Harpenden, Herts, AL5 2JQ United Kingdom (P. J. L.)

ABSTRACT

Asparagine is present in the mature leaves of young pea (*Pisum sativum* cv Little Marvel) seedlings, and is synthesized in detached shoots. This accumulation and synthesis is greatly enhanced by darkening. In detached control shoots, [^{14}C]aspartate was metabolized predominantly to organic acids and, as other workers have shown, there was little labeling of asparagine (after 5 hours, 3.1% of metabolized label). Addition of the aminotransferase inhibitor aminooxyacetate decreased the flow of aspartate carbon to organic acids and enhanced (about 3-fold) the labeling of asparagine. The same treatment applied to darkened shoots resulted in a substantial conversion of [^{14}C]aspartate to asparagine, over 10-fold greater than in control shoots (66% of metabolized label), suggesting that aspartate is the normal precursor of asparagine.

Only traces of glutamine-dependent asparagine synthetase activity could be detected in pea leaf or root extracts; activity was not enhanced by sulfhydryl reagents, oxidizing conditions, or protease inhibitors. Asparagine synthetase is readily extracted from lupin cotyledons, but yield was greatly reduced by extraction in the presence of pea leaf tissue; pea leaf homogenates contained an inhibitor which produced over 95% inhibition of an asparagine synthetase preparation from lupin cotyledons. The inhibitor was heat stable, with a low molecular weight. Presence of an inhibitor may prevent detection of asparagine synthetase in pea extracts and in *Asparagus*, where a cyanide-dependent pathway has been proposed to account for asparagine synthesis: an inhibitor with similar properties was present in *Asparagus* shoot tissue.

Asparagine is a major transport form of N in many plants, including legumes such as lupin (1) and pea (20). Asparagine synthetase (EC 6.4.5.4), catalyzing the glutamine-dependent amidation of aspartate, is active in germinating seedlings (9, 15, 17), but there is some question about the synthesis of asparagine in older, nongerminating tissue. ^{15}N-Labeling patterns in pea leaves (2, 3) and spinach (21) are consistent with the operation of asparagine synthetase, yet there are some difficulties in the acceptance of this pathway. ^{14}C-Labeled aspartate is a poor precursor for asparagine in pea roots (13) and soybean cotyledons (17). Active preparations of asparagine synthetase have been obtained only from cotyledonary tissue (9, 15, 17). Some activity was detected in corn roots (18), but presence of an aspartate-dependent synthesizing system in mature leaves has not been reported. Cyanide can be incorporated into asparagine (see 10) and enzymes of the β-cyano-alanine pathway have been detected, for example in *Asparagus* (4). However, the enzymic properties and physiological concentrations of substrates have lead a number of workers to doubt that this can be a major route for asparagine synthesis (17–19). The principal argument in favor of the cyanide pathway is the inability to detect asparagine synthetase in *Asparagus* tissue (4).

Although the level of asparagine decreases in mature pea leaves, presumably due to selective export (20), labeling data suggests that pea leaf tissue is capable of asparagine synthesis (2). This has been confirmed here by analysis of detached shoots; aspartate can be shown to be an asparagine precursor if a transaminase inhibitor is used to prevent the rapid equilibration of aspartate carbon with the organic acid pool. The presence of a potent asparagine synthetase inhibitor, detected in pea leaves and *Asparagus* shoots, may account for the inability to detect the enzyme in these and other tissues.

MATERIALS AND METHODS

Plants of *Pisum sativum* (cv Little Marvel) were grown in nutrient solution, with 4 mM nitrate, as described earlier (3) and were used when the fifth leaf reached one-half full expansion. Samples for analysis were taken after 4 h of the daily (12 h) light period. For feeding experiments, shoots were cut under water, rinsed, and transferred to solutions as described.

Lupinus albus seeds were treated 5 min with dilute hypochlorite, rinsed, soaked with aeration 10 to 14 h, and germinated 5 d in vermiculite at 25°C.

Asparagus officinalis tissue consisted of whole seedling shoots, grown from seed for 4 weeks after germination, or the expanding shoots from a rootstock, taken when branches, but not individual cladodes, were unfolding.

Analysis. Tissue was ground briefly in 3 volumes water and then further ground after addition of a solution of 5-sulfosalicylic acid (final concentration, 50 mg/ml). Samples were clarified by centrifugation, brought to pH 2, and aliquots were analyzed with a Beckman 119BL amino acid analyzer (7).

^{14}Carbon was detected by liquid scintillation counting. Labeled amino acids were estimated using the analyzer and counting effluent fractions; ninhydrin flow was considerably reduced so that peaks could be identified in the effluent but colour development did not interfere with counting.

L-[U-^{14}C]Aspartic acid was obtained from Amersham; controls showed that contamination with asparagine was negligible.

Extraction and Assay of Asparagine Synthetase. Extraction procedures were based on the method of Rognes (14, 15). Tissue was ground with 2.5 volumes extraction buffer (50 mM Tris, 0.1 mM EDTA, 2 mM $MgCl_2$, 20 mM KCl, 20% glycerol, pH 7.8; 0.5 mM DTT and 4 ml/l 2-mercaptoethanol were added immediately before use). Clarified homogenate was obtained by filtering and

[1] Supported by a grant to K. W. J. from Natural Sciences and Engineering Research Council of Canada.

centrifugation for 10 min at 20,000*g*. The fraction for enzyme assay was precipitated at 65% saturation of ammonium sulfate, and was redissolved in a small volume of extraction buffer (one-half to one-third that of the original fresh weight) and passed through Sephadex G-25 equilibrated with the same buffer.

For lupin cotyledons, the method was similar, but 4 volumes extraction buffer were used, and chopped cotyledon tissue was homogenized with a Polytron. Asparagine synthetase was recovered at 55% saturation of ammonium sulfate; the protein floated when centrifuged.

Aliquots (150 μl) of enzyme preparations were added to 200 μl assay mix (pH 8.0) plus 150 μl extraction buffer (or other addition) to give the following final concentrations: aspartate, 10 mM; ATP, 10 mM; $MgCl_2$, 10 mM; glutamine, 1 or 2 mM. Assays were performed in 1.5-ml plastic centrifuge tubes, and were incubated for up to 1 h at 30°C. Reactions were terminated by addition of 0.1 ml 5-sulfosalicylic acid (300 mg/ml) and centrifugation. Aliquots of supernatant were analyzed for asparagine content.

RESULTS

Asparagine Accumulation in Pea Shoots. Initial attempts to demonstrate the presence of asparagine synthetase in pea tissues were unsuccessful. We therefore looked for conditions under which synthesis and accumulation were clearly demonstrated, to provide a tissue for investigation of the synthetic mechanism.

Detached pea shoots were supplied with a range of possible asparagine precursors including aspartate, ammonium, and glutamine, at concentrations up to 20 mM. None of these treatments consistently increased the asparagine content during a 6-h incubation. Although asparagine has been described as a product of ammonia detoxification, the response to high ammonia was a massive synthesis of glutamine (10- to 20-fold increase over 6 h). Longer (24 h) incubations gave rather variable results, although there was some indication of asparagine accumulation in treatments containing ammonium plus aspartate, as also found by Stewart (16).

More consistent and dramatic accumulations of asparagine were found in darkened plants. After 2 to 3 d darkness, there was a considerable increase of asparagine in leaves of intact plants; this was due to synthesis in leaf tissue since the accumulation also occurred in detached shoots when darkened (Table I). Asparagine levels showed considerable variation between plants, but there was a substantial (7-fold) increase over 3 d; the increase between 24 and 72 h corresponded to a rate of 360 nmol Asn/g fresh weight·h. Other amino acids showed only minor changes. Asparagine did not increase in the light.

Asparagine Synthetase Measurements. Various attempts were made to detect asparagine synthetase activity in pea tissues—roots, whole shoots, young and old leaves, and darkened leaves which were known to be synthesizing asparagine.

Initially the assay used was based on measuring the formation of [^{14}C]asparagine, separated from the precursor [^{14}C]aspartate on small columns of Dowex 1 resin (17). However, at low levels of activity the assays were rather variable, and blank levels were affected by some additions to the assay medium. Direct determination of asparagine gave unambiguous results, and had the added advantage of indicating proteolysis activity and glutaminase activity during the assay.

Asparagine synthetase activity was low or undetectable in all pea tissues, with values typically in the range of 10 to 30 nmol/g fresh weight·h. In contrast, extract from lupin cotyledons gave activities of at least 600 to 700 nmol/g fresh weight·h.

Various alterations in extraction procedure were tried for pea leaves: use of polyethylene glycol MW8000 (18% w/v) to precipitate the enzyme; omission of sulfhydryl reagents during extraction or assay, and addition of oxidized glutathione; addition of protease inhibitors (phenylmethyl sulfonyl fluoride, 1 mM; *p*-chloromercuribenzoate, 2 mM; iodoacetate, 10 mM); addition of PVP, glutamine, or ATP during extraction. None of these treatments enhanced the activity of asparagine synthetase, although alterations in glutaminase and protease activities were seen in some assays.

Asparagine was not being lost or turned over during the assay; asparaginase activity was low, and a small amount of cold asparagine did not trap labeled asparagine when [^{14}C]aspartate was included in the assay.

Aspartate as a Precursor for Asparagine. To determine whether asparagine synthetase could be operating, the ability of aspartate to act as an asparagine precursor was investigated. Under normal conditions, relatively small amounts of aspartate (^{14}C label) are incorporated into asparagine, and much of the carbon appears in the organic acid pool. This probably results from an equilibration through transamination. Inhibition of transamination, in a situation where asparagine synthesis is enhanced, should provide an optimum situation for detection of aspartate incorporation (Table II). In all treatments (shoots from intact plants, illuminated or darkened shoots), addition of aminooxyacetate resulted in a large reduction of labeling of the organic acid fraction and retention of much of the label in the supplied aspartate. The inhibitor treatment also reduced the flow of label into some other amino acids, such as glutamate, which would be formed from the organic acid pool. In contrast, labeling of asparagine was enhanced several-fold by inhibitor treatment,

Table I. *Changes in Levels of Some Amino Acids in Detached Pea Shoots*

Pea shoots were detached and placed in one-quarter strength nutrient solution with 2 mM nitrate, in normal (12 h) photoperiod or continuous darkness. Two samples, each of two plants, were extracted for analysis at times specified. If unspecified, variation was less than 10% of the mean value.

Time	Asp	Thr	Ser	Asn	Glu	Gln
h				*μmol/g fresh wt*		
0	3.60	0.08	0.89	3.17 ± 1.06	2.58	1.31
Normal photoperiod						
24	2.79	0.29 ± 0.04	1.31	1.48 ± 0.55	2.20	0.85
48	2.58	0.42	1.61	3.68 ± 1.94	3.05	1.27
72	2.37	0.51	1.44	2.88	3.05	1.02 ± 0.17
Darkness						
24	4.19	0.17	1.44 ± 0.25	6.73 ± 1.56	4.78	0.55 ± 0.13
48	4.36	0.17 ± 0.08	1.27	18.1 ± 7.1	3.47 ± 0.59	0.42
72	3.77	0.29 ± 0.04	1.99	24.0	3.64	0.72 ± 0.13

Table II. *Distribution of Label from [^{14}C]Aspartate in Pea Shoots, and Effect of Aminooxyacetate*

Pea shoots were freshly detached, or were detached and maintained for a 48-h pretreatment in one-quarter strength nutrient solution with 2 mM nitrate, in normal (12 h) photoperiod or continuous darkness. Subsequent incubation was in normal laboratory lighting, approximately 800 lux. Treatments with aminooxyacetate (4 mM, pH 6.1) were begun 30 min before addition of label. Shoots were placed in 0.5 mM aspartate (pH 6.1) containing 2 μCi/ml L-[U-^{14}C]aspartate, with or without inhibitor. After 5 h, the shoots (two/treatment) were extracted for analysis.

Inhibitor	Detached 48 h						
	No Pretreatment		Normal photoperiod		Darkness		
	−	+	−	+	−	+ (Exp. I)	+ (Exp. II)
	% recovered cpm						
Organic acids[a]	67.6	12.5	66.9	16.4	56.0	15.0	13.1
Asp	10.9	57.0	8.4	60.3	11.4	47.8	52.0
Asn	2.7	6.9	4.2	17.2	13.3	34.3	32.2
Glu	9.8	1.0	11.1	1.8	11.1	1.7	1.4
Others[b]	9.0	22.6	9.4	4.3	8.2	1.2	1.3
Aspartate uptake, cpm × 10^{-3}/shoot	1012.1	1147.9	574.1	522.8	551.3	888.0	1040.9

[a] Eluting from amino acid analyzer soon after front; contained traces of neutral compounds.

[b] Activity predominantly in Gln and homoserine (Hse), plus materials eluted in regeneration which followed Gln/Hse; traces only in Thr and Ser.

reaching a maximum of 34% of the tissue label (66% of label metabolized from aspartate) in shoots given dark treatment. The results indicate that normal and dark-enhanced asparagine synthesis utilizes aspartic acid as a precursor. A very approximate estimation can be made for the rate of conversion of aspartate to asparagine, by using the specific activity of the aspartate pool to calculate the rate of transfer of carbon. If we assume that the specific activity rose steadily to the final value throughout the experiment (inasmuch as label was supplied continuously), then the calculated rate of transfer of carbon into asparagine is approximately 250 nmol/g fresh weight·h. A further (untestable) assumption here is that the asparagine precursor pool of aspartate is similar in specific activity to the total pool for the leaf tissue.

Asparagine Synthetase Inhibitor. If, as the above results suggest, asparagine synthetase could be functioning in the shoot tissue, the inability to detect it may be due to an extremely unstable enzyme, or its inactivation by cellular components during extraction. The latter possibility was tested by using lupin cotyledons as a known source of active asparagine synthetase.

Table III. *Inhibition of Lupin Asparagine Synthetase*

Clarified homogenates were added to asparagine synthetase assays containing enzyme equivalent to 250 mg lupin cotyledons. Asparagine production, compared to zero time controls, was assayed after 1 h. Inhibition was relative to uninhibited control values of 190 to 230 nmol Asn/h·assay.

Source of inhibitor	Equivalent Fresh Weight	Inhibition
	mg	%
Mature pea leaf		
Fresh homogenate	1.7	54
	3.3	69
	6.6	92
Boiled, 30 s	6.6	84
Boiled, 5 min	6.6	81
Dialyzed, 16 h	6.6	6
Boiled, 5 min (from shoots darkened 48 h)	6.6	65
Pea root, fresh	16.7	43
Asparagus		
Mature shoot		
Fresh	7.7	72
Boiled	7.7	75
	3.8	45
Expanding shoot, fresh	7.7	76

When an enzyme preparation (ammonium sulfate precipitate) from peas was added to lupin asparagine synthetase, no inhibitory effect was found. However, when lupin cotyledons were homogenized together with an equal fresh weight of pea leaves during enzyme extraction, the resulting preparation had only about 39% of the activity of an identical preparation made without the pea leaves. An inhibitory factor was present in the clarified homogenate of pea leaves (Table III) and was very effective when added directly to the assay. Over 90% inhibition of the asparagine synthetase equivalent to 1 g fresh weight of lupin cotyledon was caused by the extract from only 25 mg (fresh weight) of pea leaf. This inhibitor was a low mol wt compound, lost on dialysis, and could be resolved from the major protein fraction by passage through Sephadex G-25, when it appeared to coincide with the fraction containing low mol wt compounds, including amino acids. However, overnight dialysis of the ammonium sulfate precipitate from peas did not result in any increase of enzyme activity. This may indicate that the inhibitor was strongly bound, but may also reflect the instability of the enzyme: dialysis of the lupin asparagine synthetase caused a substantial (over 70%) loss of activity. The inhibitor was not inactivated by boiling and retained inhibitory activity for several days when stored at 4°C. It also showed inhibitory effect against asparagine synthetase from mung bean cotyledons. The inhibitor was also present, but slightly less active, in darkened pea shoots (Table III). A similar inhibitor was present in young seedlings and expanding shoots of *Asparagus* (Table III).

DISCUSSION

Asparagine plays a major role as transport and storage compound in pea plants. Investigation of nitrogen turnover (3) and the metabolism of asparagine (5, 12) show that, for peas at least, asparagine is not a 'dead end' product (11). Less is known of the synthetic pathway for asparagine, which is made in roots (since it is a component of xylem sap) and also in shoots, as shown here. Synthesis and accumulation appear to be under metabolic control, since a dramatic accumulation can be initiated by darkening of pea shoots (Table I). This response does not seem to be a mechanism for detoxification of ammonia, which was at normal levels in the darkened tissue. Feeding experiments showed that glutamine synthesis was the most immediate response to

high ammonia, and in fact glutamine levels were quite low in dark conditions.

The principal path for asparagine synthesis is thought to be through the action of asparagine synthetase (10), although most reports of this enzyme are in germinating seeds. There are relatively few situations where both metabolic and enzyme studies have been carried out on the same tissue. In soybean cotyledon, Streeter (17) concluded that asparagine synthetase was involved. The enzyme was present, although [^{14}C]aspartate was a poor precursor, with label more readily entering the organic acid pool. In turn, carbon from some organic acids entered asparagine, and the results were consistent with some compartmentation of the appropriate metabolic systems. A similar conclusion was reached by Mitchell and Bidwell (13) who carried out labeling studies with pea roots. Equilibration of label is a potential problem for studies involving any metabolite which is a transamination substrate (6). In the work reported here, the use of a transaminase inhibitor dramatically reduced the loss of label from aspartate to the organic acid pool. At the same time, transfer of label to asparagine was enhanced, indicating that in the pea leaf aspartate is an immediate precursor in asparagine synthesis. A calculation (admittedly made with some assumptions) of the rate of transfer of aspartate to asparagine in darkened, detached shoots, gave a figure of 250 nmol/g fresh weight·h, of the same magnitude as the measured rate of net accumulation, 360 nmol/g fresh weight·h. The labeling pattern is clearly consistent with the operation of asparagine synthetase, and is incompatible with a major participation of the β-cyano-alanine pathway in asparagine synthesis. Although enzymes of this pathway have been demonstrated in *Asparagus* (4), there are a number of objections (including the kinetic properties of the enzymes in relation to the physiological concentrations of their substrates) which suggest that the pathway is only operative in cyanide detoxification (10). The major argument in favor of the β-cyano-alanine path is the absence of asparagine synthetase in *Asparagus* tissue. The demonstration of a powerful asparagine synthetase inhibitor in *Asparagus* and pea weakens this argument and may account for the inability to detect the enzyme in some tissues. An inhibitor has also been detected in germinating mung bean seedlings (8) and explained the decline of measurable asparagine synthetase in the aging cotyledons. However, unlike the inhibitor described here, the factor from mung beans was nondialyzable, and heat labile. Investigations on the nature and role of the pea inhibitor are continuing. It will be of particular interest to determine whether the substance may play a regulatory role *in vivo*, or acts fortuitously as inhibitor only during tissue disruption. It is clear, however, that the inability to assay asparagine synthetase in plant extracts cannot be taken as certain evidence that the enzyme is inoperative in the intact tissue.

Acknowledgments—We thank Dr. J. Y. Tsukamoto for a supply of lupin seeds, and Dr. P. M. Stokes for assistance in obtaining *Asparagus* material.

LITERATURE CITED

1. ATKINS CA, JS PATE, PJ SHARKEY 1982 Asparagine metabolism—key to the nitrogen nutrition of developing legume seeds. Plant Physiol 56: 807–812
2. BAUER A, KW JOY, AA URQUHART 1977 Amino acid metabolism of pea leaves—labeling studies on utilization of amides. Plant Physiol 59: 920–924
3. BAUER A, AA URQUHART, KW JOY 1977 Amino acid metabolism of pea leaves—diurnal changes and amino acid synthesis from ^{15}N-nitrate. Plant Physiol 59: 915–919
4. COONEY DA, HN JAYARAM, SG SWENGROS, SC ALTER, M LEVINE 1980 Metabolism of L-asparagine in *Asparagus officinalis*. Int J Biochem 11: 69–83
5. IRELAND RJ, KW JOY 1981 Two routes for asparagine metabolism in *Pisum sativum* L. Planta 151: 289–292
6. JOY KW, N NISHIKAWA 1979 Cautionary note: equilibration of label in amino acids by aminotransferases. Plant Physiol 63: S-46
7. JOY KW, C SHAY, MJ MCLIMONT 1980 Effect of some organic buffers on the estimation of aspartic acid and resolution in amino acid analysis. J Chromatogr 194: 76–79
8. KERN R, MJ CHRISPEELS 1978 Influence of the axis on the enzymes of protein and amide metabolism in the cotyledons of mung bean seedlings. Plant Physiol 62: 815–819
9. LEA PJ, L FOWDEN 1975 The purification and properties of glutamine-dependent asparagine synthetase isolated from *Lupinus albus*. Proc R Soc London Ser B 192: 13–26
10. LEA PJ, BJ MIFLIN 1980 Transport and metabolism of asparagine and other nitrogen compounds within the plant. *In* BJ Miflin, ed, Biochemistry of Plants, Vol 5. Academic Press, New York, pp 569–607
11. LEES EM, KJF FARNDEN, WH ELLIOTT 1968 Studies on asparagine synthesis and utilisation in seedlings. Arch Biochem Biophys 126: 539–546
12. LLOYD NDH, KW JOY 1978 2-Hydroxysuccinamic acid: a product of asparagine metabolism in plants. Biochem Biophys Res Commun 81: 186–192
13. MITCHELL DJ, RGS BIDWELL 1970 Compartments of organic acids in the synthesis of asparagine and homoserine in pea roots. Can J Bot 48: 2001–2007
14. ROGNES SE 1975 Glutamine-dependent asparagine synthetase from *Lupinus luteus*. Phytochemistry 14: 1975–1982
15. ROGNES SE 1980 Anion regulation of lupin asparagine synthetase. Phytochemistry 19: 2287–2293
16. STEWART CR 1979 Effect of ammonium, glutamine, methionine sulfoximine and azaserine on asparagine synthesis in soybean leaves. Plant Sci Lett 46: 269–273
17. STREETER J 1973 In vivo and in vitro studies on asparagine biosynthesis in soybean seedlings. Arch Biochem Biophys 157: 613–624
18. STULEN I, GF ISRAELSTAM, A OAKS 1979 Enzymes of asparagine synthesis in maize roots. Planta 146: 237–241
19. TING IP, WC ZSCHOCHE 1970 Asparagine biosynthesis by cotton roots. Plant Physiol 45: 429–434
20. URQUHART AA, KW JOY 1981 Use of phloem exudate technique in the study of amino acid transport in pea plants. Plant Physiol 68: 750–754
21. WOO KC, JF MOROT-GAUDRY, RE SUMMONS, CB OSMOND 1982 Evidence for the glutamine synthetase/glutamate synthase pathway during the photorespiratory nitrogen cycle in spinach leaves. Plant Physiol 70: 1514–1517