Distribution and Properties of a Potassium-dependent Asparaginase Isolated from Developing Seeds of *Pisum sativum* and Other Plants[1]

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ABSTRACT

Asparaginase (EC 3.5.1.1) was isolated from the developing seed of *Pisum sativum*. The enzyme is dependent upon the presence of K^+ for activity, although Na^+ and Rb^+ may substitute to a lesser extent. Maximum activity was obtained at K^+ concentrations above 20 millimolar. Potassium ions protected the enzyme against heat denaturation. The enzyme has a molecular weight of 68,300.

Asparaginase activity developed initially in the testa, with maximum activity (3.6 micromoles per hour per seed) being present 13 days after flowering. Maximum activity (1.2 micromoles per hour per seed) did not develop in the cotyledon until 21 days after flowering. Glutamine synthetase and glutamate dehydrogenase were also present in the testae and cotyledons but maximum activity developed later than that of asparaginase.

Potassium-dependent asparaginase activity was also detected in the developing seeds of *Vicia faba*, *Phaseolus multiflorus*, *Zea mays*, *Hordeum vulgare*, and two *Lupinus* varieties. No stimulation of activity was detected with the enzyme isolated from *Lupinus polyphyllus*, which has previously been shown to contain a K^+-independent enzyme.

Asparagine is a major nitrogen storage and transport compound in plants (1, 11, 18, 19). In *Lupinus* asparagine may account for 70% of the nitrogen transported to the pod and seed in the phloem although only 7 to 10% of the residues of the seed protein are asparagine. When [U-^{14}C], amide-[^{15}N]asparagine was supplied via the phloem to developing lupin seeds the amide nitrogen was rapidly converted to a wide variety of amino acids in the seed protein (1). It was suggested that asparagine was metabolized by the enzyme asparaginase (L-asparagine amido-hydrolase, EC 3.5.1.).

Asparaginase has been purified and characterized from *Lupinus polyphyllus* (9); however, the enzyme was not found in the majority of lupin varieties tested nor in other legumes, *e.g. Pisum, Phaseolus*, or *Vicia* (8), a result confirmed by other workers in *Pisum* (2) and soya bean (23). Two other possible routes of asparagine metabolism have been considered (7); the first possibility that asparagine could substitute for glutamine in the GOGAT[2] reaction has not

been confirmed with cell free extracts from a variety of sources (2, 14, 23, 28). The second route, in which asparagine is transaminated to give α-ketosuccinamic acid which subsequently breaks down to release ammonia, has been demonstrated in soya bean (24) and pea leaves (12) but we have been unable to find asparagine transamination in maturing cotyledons of *P. sativum*. Despite this lack of positive evidence for asparagine utilization we have confirmed a previous report (16) that isolated developing pea cotyledons grown in culture can utilize asparagine as the sole nitrogen source for extensive synthesis of storage protein (10). Studies with enzyme inhibitors suggest that asparagine is first metabolized to ammonia which is reassimilated by the GS/GOGAT pathway (15). This paper reports the results of a further search for enzymic evidence for asparagine breakdown and describes the isolation, from developing seeds of *Pisum sativum* and a range of other plants, of an asparaginase which is totally dependent upon the presence of K^+ ions.

MATERIALS AND METHODS

PLANT MATERIAL

P. sativum (var. Feltham first) plants were grown in pots with KNO_3 as an N source in a greenhouse during the spring of 1978. Flowers were tagged with the date as they opened. Other plants used were grown at Rothamsted, except *Lupinus* species which were generously supplied by Mr. C. Looker of the Wolfson Research Unit, University of Reading, U.K.

ENZYME ASSAYS

Asparaginase. [U-^{14}C]Asparagine (Radiochemical Centre, Amersham) was purified by passing it in solution through a Dowex 1 column to remove contaminating aspartate and recrystallizing it from ethanol. The reaction mixture, in 1.5-ml capped polypropylene microcentrifuge tubes, contained 1.2 μmol asparagine (60,000 cpm/μmol), 6 μmol Tris-HCl buffer (pH 8.0), and 6 μmol KCl (when specified) in a final volume of 120 μl. At the start of the reaction 60 μl were withdrawn as the zero time sample and the remainder incubated at 30 C for 1 h. The assay was terminated by boiling for 5 min. The tubes were then centrifuged to remove denatured protein and 2 μl of 20% (w/v) $(NH_4)_2SO_4$ added to ensure a sharp aspartate spot after chromatography. Asparagine was separated from aspartate by chromatography on cellulose-silica TLC plates in phenol-H_2O (80:20) (27). The amino acids were located, removed from the TLC plates, and counted as described previously (4). Counts located in aspartate from zero

[2] Abbreviations: GOGAT: glutamate synthase; GS: glutamine synthetase; GDH: glutamate dehydrogenase.

time incubations were subtracted from the test reaction. Enzyme activities were expressed as μmol aspartate formed/h.

Glutamine Synthetase. This was assayed by the formation of γ-glutamyl hydroxamate in the synthetase reaction as described by Rhodes et al. (20). Reaction mixtures (1.6 ml) contained 10 μ mol ATP, 90 μmol $MgCl_2$, 10 μmol hydroxylamine, 150 μmol glutamate, and 80 μmol imidazole buffer (pH 7.2). Assays were carried out at 30 C for 20 min and terminated by the addition of the ferric chloride reagent.

Glutamate Dehydrogenase. NADH-dependent activity was measured by following the decrease in A at 340 nm in the presence of NH_4^+ plus α-ketoglutarate. The assay mixture (1 ml) contained 10 μmol of α-ketoglutarate, 50 μmol $(NH_4)_2SO_4$, 0.2 μmol NADH, 1 μmol $CaCl_2$, and 50 μmol Tricine-KOH buffer (pH 7.2). The addition of Ca^{2+} ions was essential to obtain maximum activity.

EXTRACTION AND PURIFICATION OF ASPARAGINASE

Cotyledons or testae were ground in 3 volumes of extraction buffer containing 50 mM Tris-HCl (pH 8.0), 15 mM mercaptoethanol, 1 mM phenylmethylsulfonylfluoride, 50 mM KCl, and 10% (w/v) glycerol, squeezed through muslin and centrifuged at 10,000g for 20 min at 2 C. The supernatant was slowly brought to a concentration of 0.5 g $(NH_4)_2SO_4$/ml and the precipitated protein removed by centrifugation. When total levels of asparaginase were assayed the redissolved pellet was desalted on a Sephadex G-25 column equilibrated in extraction buffer; when it was required to remove K^+ the equilibration buffer did not contain K^+. To purify the $(NH_4)_2SO_4$ precipitate further it was dissolved in buffer containing 10 mM asparagine and ethanol slowly added to a final concentration of 60% (v/v) (considerable loss of activity was observed above this value). The ethanolic extract was left at 0 to 4 C for 1 h and the precipitate removed by centrifugation. The supernatant was applied to a Sephadex LH-20 column equilibrated in extraction buffer (with or without K^+) and the asparaginase eluted free from ethanol.

EXTRACTION OF GLUTAMINE SYNTHETASE AND GLUTAMATE DEHYDROGENASE

Pea cotyledons or testae were ground in 50 mM imidazole-HCl (pH 7.2), 1 mM DTT, 0.5 mM EDTA, and 0.1% Triton X-100. The extract was squeezed through muslin and centrifuged at 10,000g for 20 min. The supernatant was passed through a column of Sephadex G-25 equilibrated in extraction buffer minus Triton X-100. The extractable protein content of the seeds was determined by the method of Itzhaki and Gill (6) and the total N by Kjeldahl analysis.

MOL WT DETERMINATION

The method described by Martin and Ames (13) was used. The extracts were layered on linear sucrose gradients of 5 to 20% (w/v) prepared with or without 50 mM KCl. The gradients were centrifuged on a Beckman L2-65B in a SW 27 rotor at 25,000 rpm for 24 h. Beef liver catalase (mol wt 250,000) was used as a standard marker.

RESULTS

Characteristics of Enzyme. The assay system used was a modification of that of Lea et al. (9). To increase the sensitivity of the assay, a radioactive rather than colorimetric determination of aspartate was used. Considerable care was taken to remove any contaminating aspartate from the [^{14}C]asparagine. After the enzyme reaction, radioactivity could only be detected in asparagine and aspartate on the TLC plate. The reaction rate was linear for 3 h at 30 C, and proportional to the amount of enzyme added.

Asparaginase was detected in the testa and cotyledon of matur-

ing pea seeds but not in the liquid endosperm. The enzyme was found to be stable and active in 60% ethanol and ethanol precipitation of other proteins was used to give a 12- to 15-fold purification of asparaginase; the ethanol was then removed by passing the extract over Sephadex LH-20. Specific activity values for the enzyme from the testa and cotyledon after ethanol purification were 31 and 1 μmol/h·mg protein, respectively.

The enzyme was only active in the presence of K^+ ions. The effect of different concentrations of K^+ on asparaginase isolated from both cotyledon and testa is shown in Figure 1. In both cases maximal activity is closely approached at 20 mM K^+ and the concentration of K^+ giving half-V_{max} were 4.5 mM and 6.0 mM for the testa and cotyledon enzyme, respectively. These values should, however, be treated with caution as they probably represent the effect of K^+ on both the activity and stability of the enzyme (Fig. 3). Various other cations were tried as substitutes for K^+ but only Na^+ and Rb^+ were able to promote activity to a significant level (Table I). A number of anions were tested in the presence of K^+ at 33 mM. Iodide, nitrate, sulfate, phosphate, α-ketoglutarate, and tartrate had no effect on asparaginase activity when compared to chloride; bromide inhibited by 11.1%. Mercuric chloride at 1 mM inhibited asparaginase activity by over 95%.

The effect of concentration of asparagine on asparaginase activity is shown in Figure 2 and K_m values were calculated as 3.2 mM for the cotyledon and 3.7 mM for the testa enzyme. The two products of the reaction, aspartate and ammonia, inhibited 33% at 6 mM and 6% at 10 mM, respectively.

In the absence of K^+, the enzyme was unstable in solution (Fig. 3). Almost 90% of activity was lost after 10 min at 30 C, and 40% lost at 20 C. Very little activity was lost in the presence of K^+ at either 20 or 30 C. Sodium was able to substitute for K^+ in its ability to prevent heat denaturation to a lesser extent, but asparagine had no action as a protective agent. This stabilizing effect of K^+ is separate from its activating effect since when assayed under conditions where the enzyme shows a reasonable degree of stability in the absence of K^+, i.e. at 20 C for 20 min, the enzyme is still inactive unless K^+ is present.

Mol Wt. The mol wt of the cotyledon and testa enzyme were very similar, and an average value of 68,300 was determined. Attempts to determine an accurate value for the mol wt of the enzyme in the absence of K^+ were unsuccessful due to rapid loss of activity. The value is very close to that determined for the K^+-independent enzyme (72,000) isolated from L. polyphyllus (9).

Changes in Asparaginase Activity during Development. During the course of this investigation Storey and Beevers (22) published a detailed account of the developmental changes that take place during the maturation of P. sativum seed. We have therefore reported only results that are necessary to show the changes in
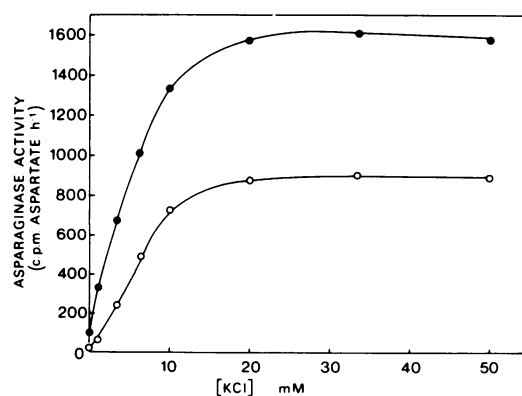

FIG. 1. Effect of increasing concentration of KCl on asparaginase activity isolated from testa (●——●) and cotyledon (○——○) of immature P. sativum seeds.

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Table I. *Activity of Asparaginase Isolated from Cotyledons and Testa of P. sativum in Presence of Various Cations*

All cations were present at 33 mM as the chloride. A Sephadex LH-20-purified extract was used as a source of enzyme.

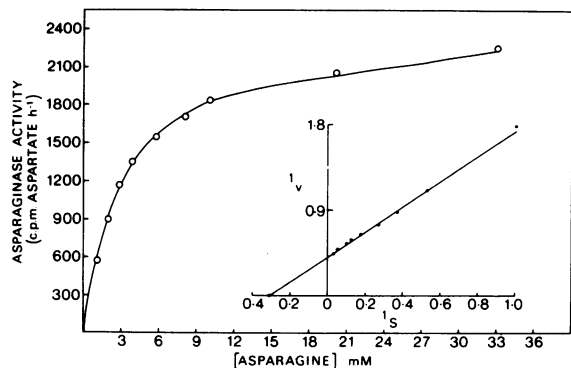| Cation	Relative Activity of Enzyme	
	Testa	Cotyledon
	%	
Potassium	100.0	100.0
Sodium	38.8	26.6
Rubidium	36.3	15.5
Ammonium	7.5	1.1
Lithium	6.3	1.6
Cesium	5.9	0
Tris	5.6	1.1
Magnesium	5.0	2.2
Calcium	3.8	0

FIG. 2. Effect of increasing concentration of asparagine on the asparaginase activity isolated from the cotyledons of immature *P. sativum* seeds. (Inset: Lineweaver-Burk plot).
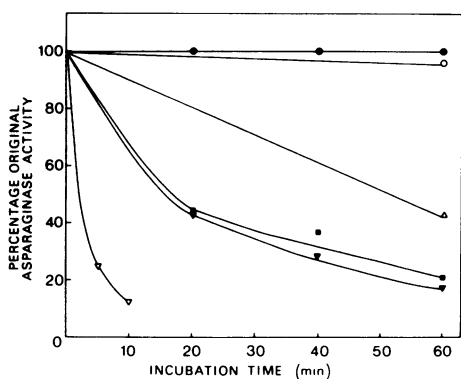

FIG. 3. Stability of testa asparaginase to heat treatment in the presence and absence of 50 mM potassium and 10 mM asparagine. (●——●): 20 C plus potassium; (○——○): 30 C plus potassium; (△——△): 30 C plus sodium plus asparagine; (■——■): 20 C no additions; (▼——▼): 20 C plus asparagine; (▽——▽): 30 C plus asparagine. The remaining activity was determined by bringing the LH-20-purified extract to 10 mM asparagine and 50 mM KCl and assaying at 30 C.

asparaginase activity in relation to other developmental processes. Our results for the time-course of fresh weight and extractable protein increase in the cotyledons (Fig. 4A) are comparable to the results of Storey and Beevers (22). When asparaginase was measured over this period (Fig. 4B) it was found that there was a rapid increase in activity in the testa immediately prior to the onset of protein synthesis in the cotyledon. Subsequently, asparaginase then appeared in the cotyledon increasing to a maximum during the period of maximum protein accumulation and then decreasing to virtually nothing at maturity.
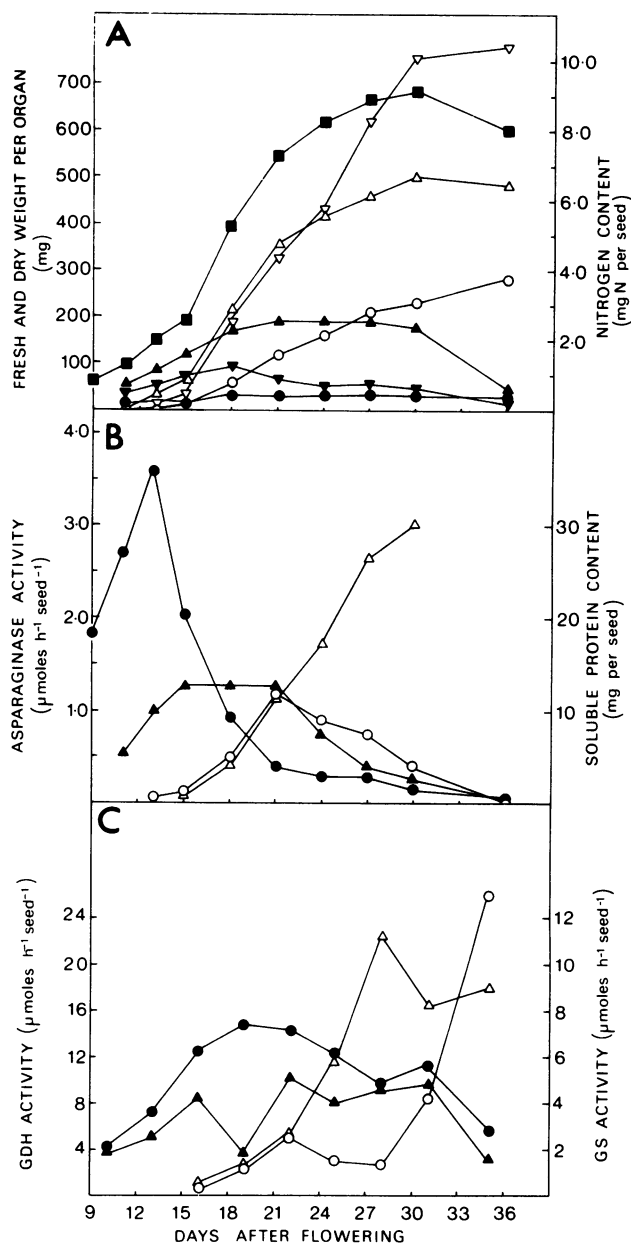

FIG. 4. A: developmental change in fresh and dry weight of testa and cotyledon during maturation of *P. sativum* seed. (●——●): Dry weight of testa; (▲——▲): fresh weight of testa; (▼——▼): N content of testa; (○——○): dry weight of cotyledons; (△——△): fresh weight of cotyledons; (▽——▽): N content of cotyledons; (■——■): fresh weight of whole seed. B: developmental changes in asparaginase and protein in testa and cotyledon during maturation of *P. sativum* seed. (●——●): Asparaginase in testa; (○——○): asparaginase in cotyledons; (▲——▲): protein content of testa (× 10); (△——△): protein content of cotyledons. C: developmental changes in GS and GDH activity in testa and cotyledons during maturation of *P. sativum* seed. (●——●): GS in testa; (○——○): GS in cotyledons; (▲——▲): GDH in testa; (△——△): GDH in cotyledons.

Asparaginase activity will produce ammonia which must be reassimilated if maximum use is to be made of the transported nitrogen. The two possible routes of ammonia assimilation (14) require either the enzyme GS or GDH. The two enzymes were monitored during maturation of the pea seed, and the developmental changes observed are shown in Figure 4C. Maximum activity of both GS and GDH in the testa was detected slightly later than the maximum activity of asparaginase. In the cotyledons there was again a lag between the development of asparaginase

activity and GDH and a further lag before the development of high levels of GS activity.

K⁺-dependent Asparaginase in Other Seeds. In a report of asparaginase in lupin seeds in which the enzyme was extracted and assayed in the absence of K⁺ ions it was found that the enzyme was restricted to a relatively small number of lupin varieties (9) and not found in certain other legume seeds (8). We have, therefore, tested these plants again for the presence of asparaginase and have studied the effect of K⁺ ions on activity (Table II). As in the previous study little or no asparaginase activity was found in the absence of K⁺ (even though a much more sensitive assay has been used) in the seed of *P. sativum, Vicia faba, Phaseolus multiflorus, Lupinus albus* (var. Buttercup) or *L. mutabilis* (var. Tabor) although in all cases significant levels of activity were found with K⁺ ions present in the assay. In contrast, asparaginase was readily demonstrated in *L. polyphyllus* in the absence of K⁺ ions (as before [9]) and was not further increased by the addition of K⁺. Asparaginase activity was also detected in the developing grains of maize and barley, which was considerably stimulated by the addition of K⁺ ions.

DISCUSSION

Although asparagine has been recognized as an important storage compound for reduced N in plants for over 170 years (3, 7, 11) it is only recently that any successful attempts have been made in understanding the enzymology of asparagine breakdown. Atkins *et al.* (1) demonstrated the presence of an asparaginase in *L. albus* and we have purified a similar enzyme from *L. polyphyllus* (9). Streeter (24) and Lloyd and Joy (12) have characterized a second route of asparagine breakdown in which asparagine is first transaminated to α-ketosuccinamic acid. However, we had been unable to demonstrate either asparagine transaminase or asparaginase activity in a number of maturing legume seeds, including *L. albus* and species known to use asparagine as a sole source of N for cotyledonary protein synthesis (10, 16, 26). The reason for this is now clear; the asparaginase in these species is strictly K⁺-dependent and in all of our previous work we have used Tris buffer without added K⁺; in contrast Atkins *et al.* (1) used phosphate buffer (cation unspecified) and therefore the K⁺ dependence of the enzyme is unknown. As the asparaginase isolated from all other seeds tested (except *L. polyphyllus*) was strongly K⁺-stimulated, it may be assumed that the enzyme is widely distributed. In contrast the *L. polyphyllus* enzyme which is completely active in the absence of K⁺ has a very narrow distribution (unpublished results). It is difficult to estimate the [K⁺] at the site of enzyme activity, but the [K⁺] in the phloem in which the asparagine is transported to the seed varies between 50 and 100 mM (19).

Even though K⁺ plays an important role in activating enzymes involved in a wide range of processes including starch and protein synthesis (5, 25), we have been unable to find any previous report of an asparaginase from any other source that is activated by K⁺ or any other cation. However, the asparaginase reaction is an example of an elimination reaction involving an enol-keto tautomer as an intermediate and Suelter (25) has shown that similar enzyme-catalyzed reactions involving such intermediates are K⁺-stimulated. The reason that the enzyme from some higher plants requires K⁺ as a cofactor for activity whereas those from certain lupin species (9), fungi, and bacteria (29) do not, is not known.

In certain K⁺-activated enzymes the cation plays an important role in maintaining the functional integrity of the enzyme (17). This may involve some discrete conformational change in the enzyme structure or the dissociation into inactive subunits in the absence of K⁺. We were unable to find any effect of K⁺ on the mol wt of pea seed asparaginase. Although we have no evidence as to the mechanism involved, the enzyme is very unstable in the absence of K⁺ (Fig. 3) showing that the cation is required for maintaining the integrity of the enzyme as well as for catalytic

Table II. *Occurrence of Asparaginase Activity in Developing Seeds*

Plant Material	Specific Activity	
	μmol/h·seed	μmol/h·g fresh wt
Pisum sativum var. Feltham first		
Cotyledons		
+ K⁺	0.66	1.81
− K⁺	0	0
Testa		
+K⁺	2.70	32.43
−K⁺	0.048	0.57
Pisum arvense		
Cotyledons		
+ K⁺	2.27	6.17
− K⁺	0	0
Vicia faba var. Minden		
Cotyledons		
+ K⁺	1.79	2.56
− K⁺	0.017	0.024
Phaseolus multiflorus		
Cotyledons		
+ K⁺	0.12	0.34
− K⁺	0	0
Lipinus polyphyllus		
Whole seed		
+ K⁺	0.48	9.75
− K⁺	0.44	8.9
L. albus var. Buttercup		
Cotyledons		
+ K⁺	0.64	4.70
− K⁺	0.01	0.08
L. mutabilis var. Tabor		
Cotyledons		
+ K⁺	0.41	2.18
− K⁺	0.03	0.15
Zea mays var. Fronica		
Seed		
+ K⁺	0.37	1.04
+ K−	0.07	0.19
Hordeum vulgare var. Julia		
Endosperm		
+ K⁺	0.01	0.35
− K⁺	0.0012	0.04

activity. For these reasons K⁺ should be routinely included in buffers for isolating the enzyme.

The changes in asparaginase activity during cotyledon maturation correlate well with the times of maximum protein synthesis; thus, the highest levels of activity in the testa and cotyledon are reached during the phase in which the protein content of these organs is increasing (Fig. 4B). The maximum level of activity of the cotyledonary enzyme is sufficient to allow the synthesis of up to 5 mg protein per cotyledon pair per day from asparagine. This compares with a maximum calculated rate of cotyledonary protein synthesis of 3.08 mg protein per seed per day between the 24th and 27th day after flowering.

The development of GS and GDH in the testa lags behind the maximum level of asparaginase activity by several days—this lag may be the reason that ammonia can accumulate in such large amounts in the liquid endosperm of legumes (60 mM in lupins [1] and 15 mM in the peas reported here). This accumulation is transient, as the liquid endosperm is rapidly absorbed by the expanding cotyledon, when the rate of protein synthesis accelerates. In the cotyledon, asparaginase and GS both have a peak of activity at the same time although GS begins to increase again as the seed matures and eventually reaches a much greater value. Although the amount of GS is not particularly great during the maximum phase of protein synthesis in the cotyledon, it is always

in excess of the level of asparaginase, suggesting that it is probably capable of assimilating the ammonia formed in this reaction. This conclusion is confirmed by inhibitor studies which show that the utilization of asparagine for protein synthesis in the cotyledon is inhibited by methionine sulfoximine, an inhibitor of GS (10). GS is unlikely to be required for the assimilation of NH_3 from nitrate reduction since nitrate reductase appears not to be present in pea cotyledons (10).

The reason for the rise in GS activity during the final stages of maturity of the cotyledon is not clear but it may be important in the synthesis of glutamine during the breakdown of the storage proteins during germination.

In contrast to previous workers (2, 23) we have found large amounts of GDH present in both the testa and cotyledons. Although inhibitor evidence is against any role for GDH in ammonia assimilation in the cotyledon, large concentrations of NH_4^+ accumulate in the liquid endosperm (although not in the testa or cotyledon), and it is possible that cells lining the endosperm may assimilate ammonia via GDH.

The demonstration of asparaginase in developing pea seeds completes the list of enzymes that may be expected to be of importance in the transfer of N from asparagine and glutamine into the amino acid-N required for protein synthesis. What has not yet been fully demonstrated, although it is implied from studies of cotyledons in culture (10, 16, 26) and from preliminary labeling studies (P. J. Lea, and W. R. Mills, unpublished results), is the ability of the cotyledons to synthesize the various carbon skeletons required for the various protein amino acids. The presence of a K^+-stimulated asparaginase in maize and barley along with GS and GOGAT (15, 21) suggests that the pathway of asparagine metabolism in the developing seed is the same in these plants as in peas, although a detailed study of the enzyme during the time course of seed development remains to be carried out.

LITERATURE CITED

1. Atkins CA, JS Pate, PJ Sharkey 1975 Asparagine metabolism—key to the nitrogen nutrition of developing legume seeds. Plant Physiol 56: 807–812
2. Beevers L, R Storey 1976 Glutamate synthetase in developing cotyledons of *Pisum sativum*. Plant Physiol 57: 862–866
3. Chibnall AC 1939 Protein Metabolism in Plants. Yale University Press, New Haven, Conn
4. Davies HM, BJ Miflin 1978 Advantage of o-phthalaldehyde for visualising ^{14}C-labelled amino acids on thin-layer chromatograms and an improved method for their recovery. J Chromatogr 153: 284–286
5. Evans HJ, RA Wilds 1971 Potassium and its role in enzyme activation. *In* Potassium in Biochemistry and Physiology. International Potash Institute, Bern, pp 13–39
6. Itzhaki RF, DM Gill 1964 A micro-biuret method for estimating proteins. Anal Biochem 9: 401–410
7. Lea PJ, L Fowden 1975 Asparagine metabolism in higher plants. Biochem Physiol Pflanzen 168: 3–14
8. Lea PJ, L Fowden, BJ Miflin 1976 Asparagine breakdown in leaves and maturing seeds. Plant Physiol 57: S-40
9. Lea PJ, L Fowden, BJ Miflin 1978 The purification and properties of asparaginase from *Lupinus* species. Phytochemistry 17: 217–222
10. Lea PJ, JS Hughes, BJ Miflin 1979 Glutamine and asparagine dependent protein synthesis in maturing legume cotyledons cultured *in vitro*. J Exp Bot 30: 529–537
11. Lea PJ, BJ Miflin 1980 The transport and metabolism of nitrogen compounds within the plant. *In* BJ Miflin, ed, The Biochemistry of Plants, Vol V. Academic Press, New York. In press
12. Lloyd NDH, KW Joy 1978 2-Hydroxysuccinamic acid: a product of asparagine metabolism in plants. Biochem Biophys Res Commun 81: 186–192
13. Martin RG, BN Ames 1961 A method for determining the sedimentation behavior of enzymes. J Biol Chem 236: 1372–1379
14. Miflin BJ, PJ Lea 1977 Amino acid metabolism. Annu Rev Plant Physiol 28: 299–329
15. Miflin BJ, PR Shewry 1979 The synthesis of proteins in normal and high lysine barley seeds. *In* D Laidman, RG Wyn Jones, eds, Cereals, Phytochemical Society. Academic Press, London, pp 239–273
16. Millerd A, O Spencer, WF Dudman, M Stiller 1975 Growth of immature pea cotyledons in culture. Aust J Plant Physiol 2: 51–59
17. Nandi DL, D Shemin 1968 δ-Aminolevulinic acid dehydratase of *Rhodopseudomonas spheroides*. J Biol Chem 243: 1231–1235
18. Pate JS 1973 Uptake, assimilation and transport of nitrogen compounds by plants. Soil Biol Biochem 5: 109–119
19. Pate JS 1976 Nitrogen mobilization and cycling: case studies for carbon and nitrogen in organs of a legume. *In* IF Wardlow and JB Passioura, eds, Transport and Transfer Processes in Plants. Academic Press, New York, pp 447–462
20. Rhodes D, GA Rhendon, GR Stewart 1976 The regulation of ammonia assimilating enzymes in *Lemna minor*. Planta 129: 203–210
21. Sodek L, WJ de Silva 1977 Glutamate synthase: a possible role in nitrogen metabolism of the developing maize endosperm. Plant Physiol 60: 602–605
22. Storey R, L Beevers 1978 Enzymology of glutamine metabolism related to senescence and seed development in the pea (*Pisum sativum* L.). Plant Physiol 61: 494–500
23. Storey R, M Reporter 1978 Amino acid metabolism in developing soybeans (*Glycine max*). Can J Bot 56: 1349–1356
24. Streeter JG 1977 Asparaginase and asparagine transaminase in soybean leaves and root nodules. Plant Physiol 60: 235–239
25. Suelter CH 1970 Enzymes activated by monovalent cations. Science 168: 789–795
26. Thompson JF, JT Madison, AE Muenster 1977 *In vitro* culture of immature cotyledons of Soya bean (*Glycine max* L. Merr). Ann Bot 41: 29–39
27. Turner NA, RJ Redgwell 1966 A mixed layer for separation of amino acids by thin layer chromatography. J Chromatogr 21: 129–132
28. Wallsgrove RM, E Harel, PJ Lea, BJ Miflin 1977 Studies on glutamate synthase from the leaves of higher plants. J Exp Bot 28: 588–589
29. Wriston JC Jr, TO Yellin 1973 L-Asparaginase, a review. *In* A Meister, ed, Advances in Enzymology, Vol 39. Wiley-Interscience, New York, pp 185–248