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Enzymology of Glutamine Metabolism Related to Senescence and Seed Development in the Pea (*Pisum sativum* L.)[1]

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ABSTRACT

The metabolism of glutamine in the leaf and subtended fruit of the aging pea (*Pisum sativum* L. cv. Burpeeana) has been studied in relation to changes in the protein, chlorophyll, and free amino acid content of each organ during ontogenesis. Glutamine synthetase [EC 6.3.1.2] activity was measured during development and senescence in each organ. Glutamate synthetase [EC 2.6.1.53] activity was followed in the pod and cotyledon during development and maturation. Maximal glutamine synthetase activity and free amino acid accumulation occurred together in the young leaf. Glutamine synthetase (*in vitro*) in leaf extracts greatly exceeded the requirement (*in vivo*) for reduced N in the organ. Glutamine synthetase activity, although declining in the senescing leaf, was sufficient (*in vitro*) to produce glutamine from all of the N released during protein hydrolysis (*in vivo*). Maximal glutamine synthetase activity in the pod was recorded 6 days after the peak accumulation of the free amino acids in this organ.

In the young pod, free amino acids accumulated as glutamate synthetase activity increased. Maximal pod glutamate synthetase activity occurred simultaneously with maximal leaf glutamine synthetase activity, but 6 days prior to the corresponding maximum of glutamine synthetase in the pod. Cotyledonary glutamate synthetase activity increased during the assimilatory phase of embryo growth which coincided with the loss of protein and free amino acids from the leaf and pod; maximal activity was recorded simultaneously with maximal pod glutamine synthetase.

We suggest that the activity of glutamine synthetase in the supply organs (leaf, pod) furnishes the translocated amide necessary for the N nutrition of the cotyledon. The subsequent activity of glutamate synthetase could provide a mechanism for the transfer of imported amide N to alpha amino N subsequently used in protein synthesis. *In vitro* measurements of enzyme activity indicate there was sufficient catalytic potential *in vivo* to accomplish these proposed roles.

During the course of fructification in *pisum sativum*, substantial amounts of reserve proteins are deposited over a brief period of time in the developing cotyledons (3, 5, 25). The synthesis of protein reserves creates a demand for the necessary amino acid precursors. This demand is met mainly by amino acids synthesized *de novo* utilizing reduced C and N imported by the seed (22, 37). Most of the N translocated to the ripening fruit is in the amides, glutamine and asparagine (2, 34, 37) and they are the main N donors for *in situ* synthesis of protein amino acids in the seed (19, 22). The importance of amides in the N nutrition of the ripening ovule is substantiated by the findings that asparagine and (especially) glutamine stimulate growth and protein synthesis in cultures (*in vitro*) of legume cotyledons and various plant embryos (26, 48).

The subtending leaf (leaflets plus stipule) in the reproductive node and pod (carpel wall) surrounding the ripening ovules are the most important supply organs contributing the bulk of the reduced N imported by the developing cotyledons (19, 21, 35). Pate *et al.* (38) have stated that the role of the leaf in cycling solutes to its developing fruit is second only in importance to its role in photosynthetic C fixation. The pod is almost totally committed to the N nutrition of its developing ovules (16, 27, 36, 40).

In a previous communication, we demonstrated the presence of proteolytic activity capable of releasing amino acids from the protein of aging leaves and pods (46). In this paper we report the presence of GS[3] (EC 6.3.1.2) and GOGAT (EC 2.6.1.53) activity in aging supply organs and developing recipient organs of the pea. The respective catalytic potentials of these enzymes to incorporate N into translocated glutamine and subsequently utilize this N for assimilatory growth in the cotyledons are crucial to the N economy of the plant.

MATERIALS AND METHODS

Plants (*P. sativum* L. cv. Burpeeana) were grown under controlled environmental conditions and the age (days postanthesis) of individual organs was followed as before (46). All studies were conducted with the leaf (leaflets plus stipule) and subtended fruit (pod and cotyledons) of the lowest reproductive node of each plant. Organs were harvested, dissected and combined, and determinations of fresh weight, protein, Chl, and soluble α-amino N were made as described previously (46). Values given for these determinations were confirmed by triplicate analysis of six separately prepared samples.

Glutamine Synthetase. Extracts were prepared by a modification of the method of O'Neal and Joy (29). All steps were carried out at 5 C or less. The extraction medium was 50 mM PIPES buffer (pH 6.8) containing 0.33 M sorbitol, 2 mM $NaNO_3$, 10 mM 2-mercaptoethanol, 1 mM $MnCl_2$, 2 mM EDTA, 2 mM sodium arsenate, 4 mM sodium ascorbate, and 0.1% (w/v) BSA. One g of tissue was added to 4 ml of extraction medium and then completely macerated with a mechanical razor blade chopper (4). The resulting mixture was filtered through Miracloth (Calbiochem) into a conical test tube and centrifuged at 600*g* for 10 min. The post-600*g* supernatant was collected and the chloroplast-enriched pellet was resuspended in the original extraction medium minus sorbitol, plus 0.1% Triton X. The supernatant and chloroplast fractions were filtered through a Sephadex G-50 gel column (1.5 × 20 cm) as described previously (46). In some cases, the Miracloth filtrate was applied directly to the G-50 column. Samples were eluted from the column with the extraction medium minus sorbitol and

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[3] Abbreviations: GS: glutamine synthetase; GOGAT: glutamate synthetase; PIPES: piperazine-N,N'bis(2-ethane sulfonic acid) monosodium salt, monohydrate; GHA: gamma glutamyl hydroxamate.

BSA. The protein-rich fractions, which eluted with the void volume, were pooled, analyzed for soluble amino acids (see ref. 46), and assayed for GS activity.

GS, unless stated otherwise, was measured as glutamyl transfer activity by a modification of the methods of O'Neal and Joy (30) and Varner and Webster (49). The standard transfer assay mixture contained 30 mM L-glutamine, 0.25 mM ADP, 1.33 mM EDTA, 25 mM hydroxylamine (NH_2OH, prepared fresh daily and adjusted to neutrality before use), 12 mM sodium arsenate, and 1.5 mM $MnCl_2$ in 40 mM imidazole buffer (pH 6.8). The reaction was initiated by addition of 50 to 100 μl of enzyme extract (to a final volume of 3 ml) and incubated under standard conditions at 30 C, pH 6.8, for 5 min. Catalysis was terminated by addition of 0.5 ml of cold 24% (w/v) trichloroacetic acid and 10% (w/v) $FeCl_2$ in 2.5 N HCl. Precipitated protein was immediately removed from the mixture by centrifugation at 5,000*g* for 10 min. The clarified supernatant was analyzed for the presence of GHA by spectrophotometric determination (540 nm) of its ferric chelate (8, 43). Control reactions of active or denatured (100 C, 15 min) enzyme only, or of substrate only, were similarly incubated and analyzed. A standard curve was prepared from authentic GHA (Sigma) added to a control reaction mixture and treated as the experimental. Extinction values obtained were in agreement with those reported by others (11, 43). Synthesis of GHA during the course of the reaction (as determined from the standard curve) was taken to indicate GS activity. One unit of activity is defined as the amount of enzyme which catalyzed the formation of 1 μmol of GHA · min^{-1} under the standard conditions described above. Specific activity is units · mg^{-1} of protein in the enzyme extract.

The pH optimum for glutamyl transfer activity by GS was determined by adjusting the initial pH of the incubation mixture and assaying under otherwise standard conditions. The reaction mixture was 40 mM sodium citrate-phosphate (pH 3–5) or 40 mM Tris-MES (pH 5–6, 8–9) or 40 mM imidazole (pH 6–8) buffer containing saturating levels of substrate as described above. Control assays containing only enzyme or substrate were also conducted over the pH range of 3 to 9.

Extracts from pods were also assayed for GS activity by the biosynthetic method described by O'Neal and Joy (28) except diethylenetriamine pentaacetate (DTPA) was replaced by EDTA in the reaction mixture.

Direct evidence for the synthesis of glutamine by leaf and pod extracts was obtained by a modification of the method described by Webster (52) using a reaction mix similar to that of the biosynthetic assay (28). Enzyme extracts from the G-50 column were incubated in 0.1 M Tricine-KOH, 20 mM $MgSO_4$, 1 mM EDTA, 20 mM L-glutamate, 20 mM NH_4Cl, 10 mM ATP, and 8 mM 2-mercaptoethanol (final vol, 1 ml) at pH 7.8, 30 C, for 20 min. Similar incubations were made with reaction mixtures minus glutamate, NH_4Cl, or ATP. The synthesis reaction was terminated by addition of 4 ml of ice cold acetone and the precipitated protein was removed by centrifugation. An aliquot of the resulting supernatant was applied to Whatman 3MM filter paper and the components were separated by descending flow of 80% phenol in water (v/v). The chromatograms were developed for qualitative evidence of enzyme activity as described previously (6).

Protein was determined by the method of Lowry *et al.* (23). Chl was measured in 80% (v/v) acetone by the method of Arnon (1).

Glutamate Synthetase. Enzyme extracts were obtained by procedure B described previously (6) except tissue was homogenized in a Polytron homogenizer (setting 3, 10 sec) and the grinding medium was 100 mM HEPES (pH 7.5) containing 0.1% BSA, 2 mM 2-mercaptoethanol, 2 mM EDTA, and 0.4 M sucrose. Enzyme activity was measured as before (6), except 50 to 100 μl of 100,000*g* supernatant was assayed. One unit of GS activity is the amount of enzyme which catalyzes the oxidation of 1 nmol of NADH · min^{-1} at pH 7.5, 30 C. Specific activity is units · mg of $protein^{-1}$ in the enzyme extract. Protein was determined on trichloroacetic acid precipitates by the method of Lowry *et al.* (23). Purity of substrate amino acids used in all enzyme assays was determined by paper chromatography.

Developmental Changes of Enzyme Activities. Catalytic activities reported for each developmental stage of individual organs were measured under experimentally determined optimum conditions. Reaction velocities were taken from the initial linear portion of reaction curves where catalysis was zero order with respect to substrate and first order with respect to time and amount of enzyme extract in the reaction mixture. Under these conditions, the concentration of enzyme in the crude tissue extract was assayed quantitatively in terms of catalytic effects and results were confirmed by triplicate assays on each of three separately prepared samples. Data quoted are the mean of these nine determinations.

Mixing Experiments. Two enzyme extracts (prepared as described above for GS or GOGAT) containing different levels of enzyme activity and from separate developmental stages, were brought to equal protein concentrations (protein determined by the method of Warburg and Christian [51]) then mixed in varying proportions. Samples containing 0, 25, 50, 75, and 100% of each extract were assayed for enzyme activity as described above.

RESULTS

Growth and Development. Growth patterns (fresh wt) of the leaf (leaflet plus stipule), pod (carpel wall), and cotyledons of the first blossom node of the pea are represented in Figure 1. The compound leaf was normally one-third maximum expansion at anthesis and was fully expanded at day 9. The pod reached maximum length (7.2 cm) by day 6 and was filled with fully distended seeds by day 21. Most ovules in the young carpel began to develop, but about 37% (mostly terminal embryos) were aborted, leaving five to six seeds per ripened fruit (day 33). The endosperm of the ovule was consumed by the aggressive development of the cotyledon by day 15.

In general leaf maturation preceded that of the pod, and both organs lost weight during the period of intensive cotyledonary growth (days 9–27). By day 36, the fruit had dehisced exposing the dry mature seeds, and the subtending leaf was withered and yellow.

Changes in Biochemical Constituents during Aging. Figure 2 represents the developmental changes in Chl, protein, and soluble α-amino N (amino acids) in the leaf, pod, and cotyledon. Early

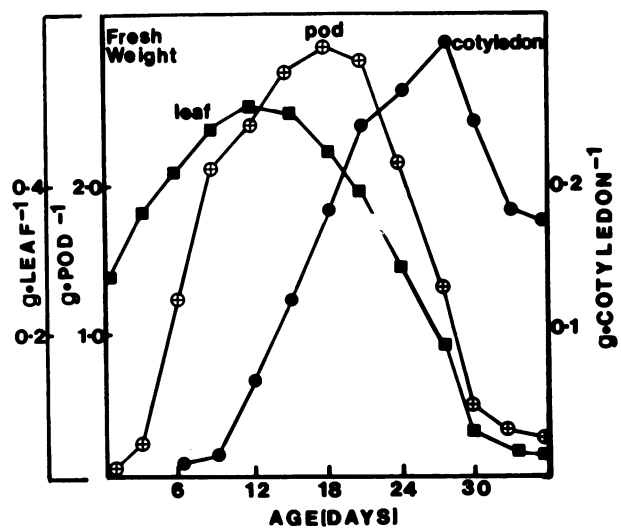

FIG. 1. Changes in fresh weight of leaf, pod, and cotyledon during development and senescence. Values are for the leaf (leaflets plus stipule) and subtended pod (carpel wall) and cotyledons of the first bloom node of the nonnodulated pea plant. Age is days postanthesis.

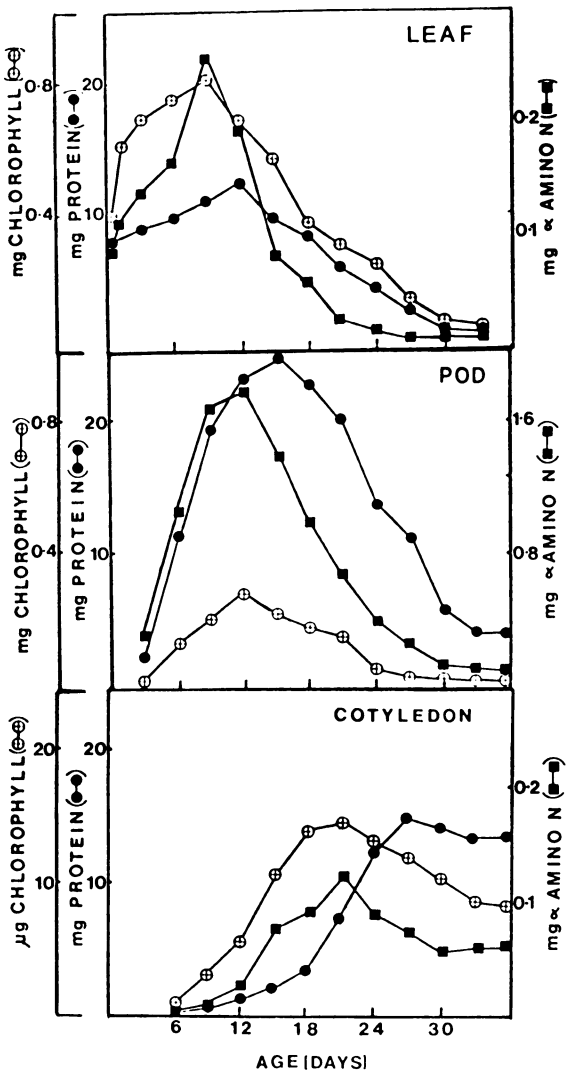

FIG. 2. Developmental changes in Chl, protein, and soluble α-amino N levels in the leaf, pod, and cotyledon of the pea. Results are expressed on a per organ basis. Age is days postanthesis.

increases in leaf (days 0–9) and pod (days 3–12) Chl were recorded, but the level decreased after full expansion of each organ. Protein content in the leaf and pod increased until days 12 and 15, respectively, then decreased while protein accumulated in the developing cotyledons (days 12–27). During expansion, free amino acids accumulated in the leaf (day 0–9) and pod (days 3–12) but disappeared rapidly from each organ as free amino acid and protein content increased in the cotyledons. The free amino acid content of the pod was consistently 8- to 12-fold that of the subtending leaf. Soluble amino acids accumulated during the first 15 days of extensive protein deposition in the cotyledon.

Enzyme Activity. The optimum conditions for measuring GS transferase activity in leaf and pod extracts were determined. Transferase activity was linear with time (up to 8 min) and the amount of enzyme extract assayed (up to 200 μl). Optimal transfer activity occurred at pH 6.8. The capacity of the crude extract to catalyze the synthesis of glutamine from L-glutamate was demonstrated by paper chromatography of the reaction products (Fig. 3). No synthesis of glutamine was detected when NH_4Cl, ATP, or L-glutamate was absent from the assay mixture.

The biosynthetic activity of GS (28) in crude pea leaf or pod extracts was also measured. In this assay, the formation of GHA from L-glutamate and hydroxylamine was linear with time and the amount of enzyme extract added to the reaction mixture at pH 7.8, and 30 C. Determinations of GS activity by the transfer method were consistently more sensitive (9- to 10-fold) than those by the biosynthetic method. O'Neal and Joy (30) reported a similar 7- to 8-fold differential sensitivity with purified GS from pea leaves. In addition, the cellular distribution of enzyme activity could be measured by either method with similar (ratio) results (29). Other workers have compared the relative activities of the two assays and found that their ratios remained constant either during purification steps (7, 8, 49), as a function of time by crude and purified preparations (32) or as a function of growth on various N sources (41). Attempts to separate the two activities in plant extracts have not been successful (7).

The optimum conditions for measuring GOGAT activity have been reported previously (6). Activity was linear with time and amount of extract assayed from each developmental stage of the pod or cotyledon considered.

Developmental Changes of Enzyme Activities. The changes in the level of extractable leaf and pod GS activity are shown in Figure 4. Activity was detected by the transfer assay because of its greater sensitivity and ease of measurement; it is also less subject to interference by contaminating enzymes in crude cell extracts (45). Developmental activity is reported as a function of fresh weight and specific activity to facilitate comparison to other studies. As suggested previously (46), it is more meaningful to consider developmental data as a function of a constant parameter that does not itself fluctuate with time. Emphasis in this paper will be on data expressed on a per organ basis.

Leaf GS activity increased with leaf expansion (to day 9) and reached a maximum at day 12 (46.5 units · $organ^{-1}$) (Fig. 4). Thereafter, activity decreased steadily until day 24, then slowly through day 33. Average activity over the 33-day developmental period of the leaf was 22.6 units $organ^{-1}$ · day. The developmental changes in leaf Chl level (Fig. 2) and GS activity (Fig. 4) were similar and the per cent of activity associated with Chl-enriched fraction of crude leaf extracts was relatively constant (24–31%) until final senescence (day 30) of the organ when it rose to 45%. This degree of association of pea leaf GS activity with the chloroplast is in agreement with that reported by O'Neal and Joy (28). Activity increased simultaneously with the level of free amino acids in the young leaf and then both decreased at near equal rates

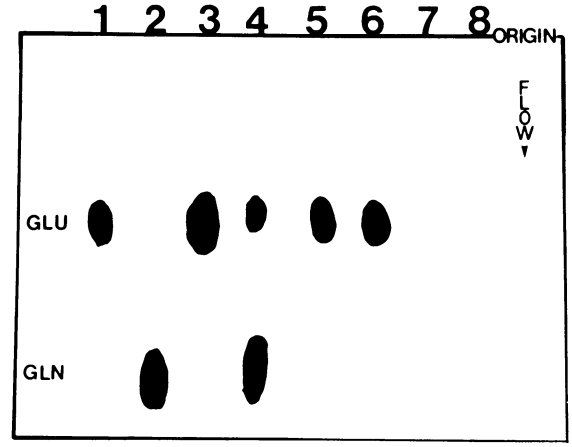

FIG. 3. Paper chromatography of the reaction products from assays for GS activity in crude extracts. Extracts of leaves (12 days postanthesis) were prepared and assayed as described in the text. Similar results were obtained with extracts of 18-day pods. The complete assay mixture was enzyme extract in 0.1 M Tricine-KOH buffer containing 20 mM $MgSO_4$, 1 mM EDTA, 10 mM ATP, 8 mM 2-mercaptoethanol, 20 mM L-glutamate, and 20 mM NH_4Cl. 1 = L-glutamate (GLU) standard; 2 = L-glutamine (GLN) standard; 3 = complete assay, 0 min; 4 = complete assay, 20 min; 5 = complete assay minus NH_4Cl, 20 min; 6 = complete assay minus ATP, 20 min; 7 = complete assay minus L-glutamate, 20 min; 8 = enzyme extract in buffer only, 20 min.

during leaf senescence (Figs. 2 and 4). Specific activity and activity · g^{-1} fresh weight were relatively constant throughout development with a slight peak at day 12 followed by a constant slow decrease until day 33 (Fig. 4).

Pod GS activity increased rapidly during early growth, then reached a maximum (48.1 units · $organ^{-1}$) 12 days after elongation ceased (Fig. 4). Thereafter, activity was rapidly lost until day 30. Average activity throughout the 33-day maturation period was 26.1 units · day^{-1}. In the pod 37 to 40% of the GS activity was associated with the chloroplast-enriched fractions of crude extracts until final senescence when it rose to over 55%. Maximum activity was recorded 6 days after Chl had begun to disappear steadily from the organ (Figs. 2 and 4). A rapid loss of the soluble amino acids from the pod also began 6 days prior to maximum activity (Figs. 2 and 4). There was little fluctuation in specific activity or activity · g^{-1} fresh weight during development with a slight decrease during senescence of the pod (Fig. 4).

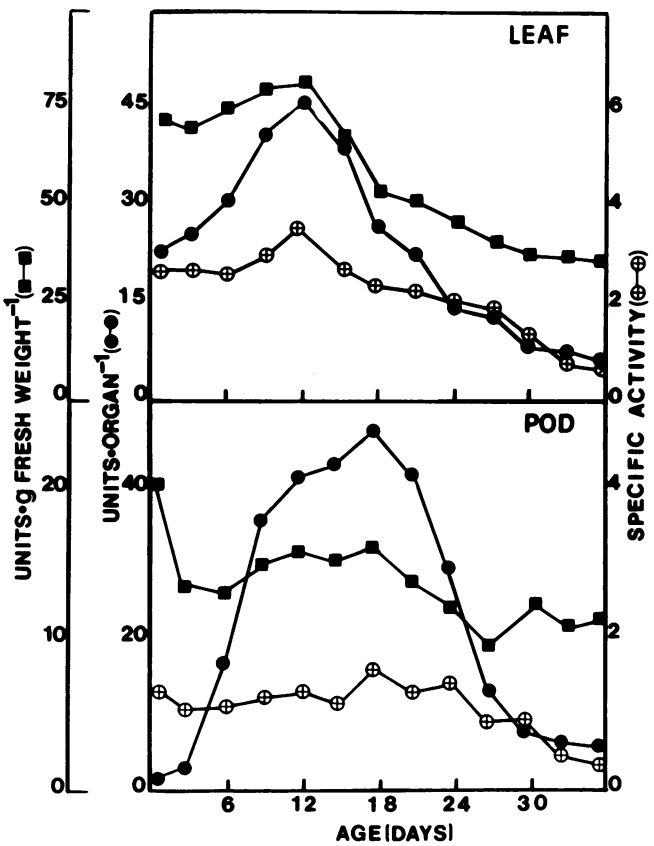

FIG. 4. Levels of GS activity in extracts from developing pea leaf and pod tissue. Transfer activity was assayed under optimum conditions as described in the text. Age is days postanthesis. Organs were harvested from the first fruiting node only. Leaf = leaflet plus stipule; pod = carpel wall. Units = μmol of GHA formed · min^{-1} at pH 6.8, 30 C. Specific activity = units · mg of $protein^{-1}$ in reaction mix.

Numerous regulatory mechanisms are known to influence GS activity in plant extracts (15, 31). It was possible that extracts from separate developmental stages of the leaf or pod contained components which influenced individual *in vitro* assays for transfer activity. Evidence against this possibility was obtained by mixing extracts from separate developmental stages which exhibited different levels of GS activity. Assays on these mixtures yielded activities which were close to the average of the individual extracts (Table I), thus indicating that no apparent regulator of GS activity was present in either extract.

GS activity was also detectable in extracts from developing cotyledons (Table II) but at a much lower level than in leaf or pod extracts (Fig. 4). Cotyledonary GS activity increased slightly during development and was highest in dry, mature seeds (0.29 units · $cotyledon^{-1}$). This level is similar to that detected in lupine seeds by Lea and Fowden (18). O'Neal and Joy (28) found that pea seeds contained only 14% of the level of activity present in leaves. Historically, pea seeds have been utilized as a source of GS for purification and characterization of the plant enzyme (8, 15, 49). These workers began with large quantities of seeds and recovered equally low or lower levels of specific activity in crude extracts as reported here.

GOGAT activity was detected in extracts of developing pea pods (Fig. 5). Activity increased rapidly as the pod elongated and continued to increase to a maximum of 201 units · pod^{-1} at day 12. Thereafter, activity decreased through day 27 when it leveled off, and was gradually lost. Average activity was 88 units · $organ^{-1}$ · day during the life of the pod. The level of activity followed changes in protein and free amino acid content in the pod (Figs. 2 and 5). Maximum pod GOGAT activity was recorded on the same day as that of leaf GS, but 6 days prior to the maximum of pod GS (Figs. 4 and 5). The specific activity of GOGAT in the pod extracts increased through day 12 and remained high for 12 days before falling rapidly. Activity based on fresh weight was relatively constant throughout development (Fig. 5).

Cotyledonary GOGAT activity increased rapidly during the period of active growth, reached a maximum (112 units · $organ^{-1}$) at day 21, and then decreased rapidly through day 33 (Fig. 5). Average activity was 43 units · $cotyledon^{-1}$ · day through development and maturation. Free amino acids and then reserve proteins accumulated as GOGAT activity increased in the developing cotyledon (Figs. 2 and 5). Maximum GOGAT activity was recorded 3 days after maximum GS activity in the pod and 9 days after that in the leaf (Figs. 4 and 5). The initial increase on a per organ basis in cotyledonary GOGAT activity is similar to the initial increase of GS activity in both leaf and pod (Figs. 4 and 5). The specific activity of GOGAT increased tremendously from day

Table I. Glutamine synthetase mixing experiments.

Enzyme extracts of equal protein concentration were prepared from each age tissue as described in the text. The extracts were combined in the indicated proportion and assayed under standard conditions. Values given are the mean of triplicate assays from two independently prepared extracts.

Leaf			Pod		
% of extract in assay mix		Specific	% of extract in assay mix		Specific
Day 12	Day 30	Activity[1]	Day 18	Day 30	Activity[1]
100	0	3.8	100	0	1.9
75	25	3.0	75	25	1.7
50	50	2.4	50	50	1.4
25	75	1.7	25	75	1.1
0	100	1.2	0	100	0.9

[1] μmol GHA formed . min^{-1} . mg protein.

6 to 12 in the developing cotyledons but decreased rapidly as reserve protein accumulated (Figs. 2 and 5). Activity on a fresh weight basis declined rapidly with the loss of specific activity (Fig. 5).

Mixing experiments were also conducted with extracts of different age organs exhibiting contrasting levels of GOGAT activity. These combined extracts produced intermediate levels of activity corresponding to the amount of each extract in the reaction mixture indicating the results of the developmental study on the enzyme activity were not influenced by an extracted, active modulator of catalysis.

Nitrogen Economy during Ontogeny. Calculations were made to determine the amount of protein N gained and lost in each organ (Table III). Each ripe fruit contained five to six mature seeds (average of 11 cotyledons · fruit^{-1}) representing a total of 143 mg of cotyledonary reserve protein (22.9 mg N)/fruit. Each pod lost 22 mg of protein (3.5 mg of N) during senescence. If, as reported, all of the protein lost from the pod was exported to the developing ovules in the fruit (16, 36, 40) it would constitute 15.3% of the N in the mature cotyledons. Recently, Pate *et al.* (36) demonstrated that 16% of the lupine seed's N had been mobilized from the senescing pod. Ten mg of protein was lost from the leaf. It is evident that there was insufficient protein N assimilated in, then mobilized from, the leaf and pod of the lowest fruiting node to account for all of the protein N deposited in the five to six seeds of the fruit.

Enzymatic Potential during Ontogeny. Calculations based on *in vitro* measurements of enzyme activity (Figs. 4 and 5) were made to assess the *in vivo* catalytic potential for glutamine metabolism during the life of each organ (Table III). These estimates may be compared to actual (*in vivo*) gain and loss of protein N, in the aging organ (Fig. 2 and above). Leaf GS activity (*in vitro*) was sufficient to have previously assimilated all of the N into glutamine which was subsequently metabolized, then incorporated into the accumulated protein of the cotyledon (days 0–12). There was also ample potential in pods to assimilate into glutamine all of the amino N which was released from leaf protein during senescence (days 13–33). Moreover, leaf GS activity (*in vitro*) during the life of the subtending fruit was adequate to have assimilated into glutamine (for export) all of the N imported by the fruit and present in the protein and amino acids of the fruits (pod plus six seeds) at senescence (day 33).

The activity of pod NAD(P)H-dependent GOGAT (*in vitro*) was sufficient (28-fold) to account for the metabolism of all glutamine amide N incorporated into pod protein (Table III). It is recognized that a ferredoxin-dependent system of GOGAT (such as occurs in the leaf, see ref. 24) might have operated in the pod. However, there was relatively little Chl in the organ (Fig. 2) and it has been demonstrated that other metabolic pathways dependent on light energy are inefficient in the pod or different from that in the leaf (12, 14, 39). Because of the quantity of the NAD(P)H-dependent enzyme in the pod, assays for activity re-

TABLE II. Glutamine synthetase activity in developing pea cotyledons.

Activities were linear with time and amount of enzyme extract assayed. Values are the mean of duplicate assays on two (day 6, 12, 24, 30, mature) or four (day 18, 24) separately prepared extracts. Mature cotyledons were taken from dry, dehisced fruits, 36 to 40 days old (post anthesis).

(Days)	6	12	18	24	30	Mature
Units per Cotyledon[1]	ND[3]	0.01	0.106	0.138	0.248	0.292
Specific Activity[2]	ND	0.011	0.028	0.011	0.018	0.023

[1]μmol GHA . min^{-1}. [2]Units · mg^{-1} protein in assay. [3]Not detected.

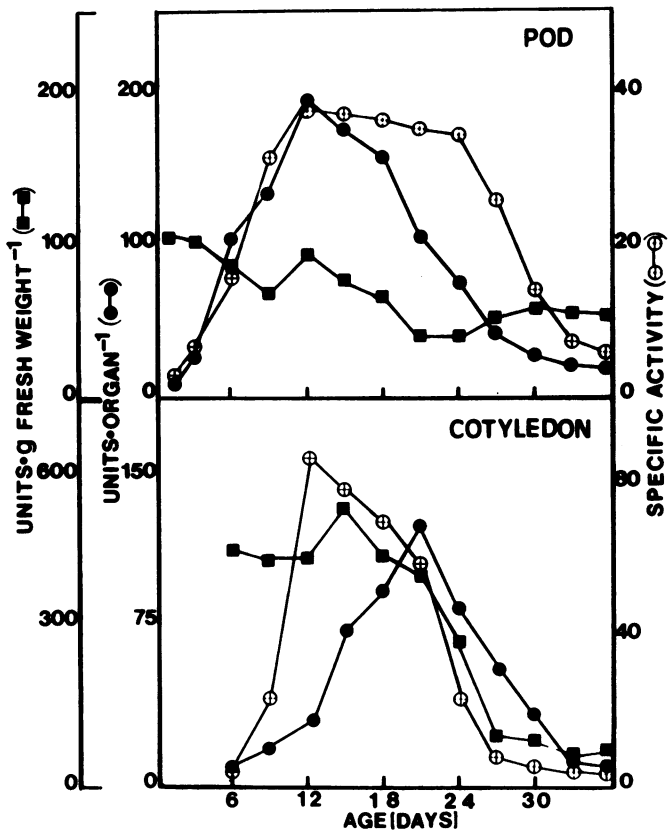

FIG. 5. Levels of GOGAT activity in extracts from developing pea pods and cotyledons. Activity was measured under optimum conditions as described in the text. Age is days postanthesis. Units = nmol of NADH oxidized · min^{-1} at pH 7.5, 30 C. Specific activity = units · mg of protein^{-1} in reaction mix.

TABLE III. Nitrogen metabolism in the aging leaf, pod and cotyledon.

Leaf, pod and cotyledon protein N content was calculated by: mg protein x 16 % = mg protein N. Final protein N (day 33) was subtracted from the maximum (Max) protein N (Figs. 4 and 5) to determine loss of protein N from each organ (Fig. 2). Glutamine synthetase (GS) activity was calculated from the biosynthetic assay. GS values are quoted as mg amide N potentially incorporated into glutamine . organ^{-1} over the developmental period (day 0-12, 13-33, total) indicated. Glutamate synthetase (GOGAT) activity was determined spectrophotometrically, where 1 nmol NADH oxidized = 1 nmol glutamine consumed = 2 nmol glutamate produced. GOGAT activity is expressed as mg amide N potentially metabolized . organ^{-1} over the developmental period (day 1-12, 13-33, total) indicated. See Figures 4 and 5 for developmental profile of enzyme activities. To account for the difference in growth chamber vs. assay temperature, the activity of each enzyme was reduced by a Q_{10} = 2 and calculations are based on a 16 hr photoperiod. Nitrogen content in free amino acids was not included in the calculations to determine the N budget of each organ because it represented ≥ 1 % of the protein N.

Leaf						Pod								Cotyledons[1]		
Protein N Content			GS Activity			Protein N Content			GS Activity			GOGAT Activity			Protein N Content		GOGAT Activity
Max	Final	Loss	Day 0-12	Day 13-33	Total	Max	Final	Loss	Day 1-12	Day 13-33	Total	Day 1-12	Day 13-33	Total	Max	Final	Total
								mg N									
1.84	0.24	1.6	344	406	750	4.2	0.7	3.5	246	636	882	45	73	118	25.3	22.9	257.4

[1]Total for all cotyledons in the fruit.

quiring ferredoxin were not conducted in this developmental study.

In the developing cotyledons, there are sufficient (10-fold) *in vitro* catalytic potential for all of the N present in reserve proteins (*in vivo*) to have been previously metabolized through the activity of cotyledonary GOGAT (Table III).

DISCUSSION AND CONCLUSIONS

The changes on a per organ basis in leaf GS activity during ontogeny appear to coincide with changes reported for respiration, photosynthesis, and other metabolic activities in aging pea leaves (9, 34). It has been shown that photosynthetic capacity reached a maximum just after full expansion of the pea leaf (9, 10) and, in supply leaves of the reproductive node, could be maintained at near maximum for several days (34). Others have reported an accumulation of free amino acids in the young, expanding field pea leaf and have found the reduction of nitrate in the leaf was most active during this period of growth (33, 34). Wallace and Pate (50) speculated that the amides may be the immediate organic products of nitrate reduction and later, Pate *et al.* (38) found that the biosynthesis of amides in leaves was closely related to the reduction of nitrate. Glutamate to glutamine conversion has been shown to be the major mechanism of utilization of ammonia synthesized in chloroplasts (20, 24) and the capacity to use this assimilated N for subsequent amino acid biosynthesis in the leaf has been reported (19, 22, 38). Further evidence in support of a relationship between N-reducing enzymes and GS in the leaf has been discussed by Miflin and Lea (24). The accumulation of free amino acids and increasing GS activity in the young pea leaf reported here (Figs. 2 and 4) is in harmony with the observations cited above and supports the contemporary proposal that GS is involved in the physiological mechanism of N assimilation in the leaf (see ref. 24 for a review). This may be a major role of GS in the pea leaf.

We found that *in vitro* activity of leaf GS exceeded the *in vivo* requirements for reduced N in leaf protein synthesis (Table III). It is likely that glutamine produced in excess of the metabolic requirements in the supply leaf is rapidly exported as amide to the developing fruit in the same node.

Free amino acids did not accumulate during the time of protein degradation in the senescing supply leaf (Fig. 2) because they were metabolized and then exported out of the aging organ (38, 46). Speculation in support of this scheme was provied by Lea and Fowden (18) who suggested that free ammonia could arise from oxidative deamination of amino acids released during proteolysis in senescing leaves. Due to its high affinity for ammonia, GS could then readily produce glutamine for direct export or subsequent amide metabolism. Because it is known that the amides represent the bulk of translocated N and that their concentration in the phloem increases with leaf age (22, 35, 37), it might be expected that leaf GS activity would also increase during senescence. This was not the case (Fig. 4). However, *in vitro* estimates of endogenous GS activity indicate that there was an excess catalytic potential present in the aging leaf (days 13–33) to metabolize all of the leaf protein hydrolysates to glutamine (Table III). We suggest that this is another role of GS in the pea leaf.

GS activity in the pod was probably not involved in the assimilation of ammonia synthesized in the chloroplasts. Other workers have reported that little or no nitrate was presented to the aging lupin pod (36, 37) because it was reduced in the subtending leaf. Schlesier and Muntz (44) found low levels of bean pod nitrate reductase activity that decreased as the fruit aged. In contrast, maximum GS activity of the pea pod (Fig. 4) was recorded during senescence in the organ (Fig. 2).

The most likely role of pod GS is the assimilation of N released in the fruit through deamination of imported amides or oxidative deamination of amino acids released from pod protein. It has been demonstrated that the decrease in pod protein is associated with an increase in proteolytic activity (46). This increase occurred at the same time as the increase in pod GS activity reported here (Fig. 5). *In vitro* estimates of pod GS activity indicate an excess of *in vivo* potential to metabolize the N released during proteolysis (Table III).

A mechanism for the transfer of imported glutamine amide N to α-amino N is provided by the activity of GOGAT. Activity in the pod increased to its maximum during the time of rapid growth and assimilation in the organ, but prior to rapid development of the cotyledons. This period of pea ontogeny was also marked by the maximum activity of leaf GS. It is likely that during this time of growth (when the pod represents the main sink for solute exported from vegetative organs to the fruit [27]) the amide synthesized in excess in the leaf could be translocated to the pod and metabolized by GOGAT. This amino N could then be utilized in pod protein synthesis which also shows a marked increase during this period (Fig. 2). Additionally, it is possible that glutamine and GOGAT in the pod operate in an associated manner in the assimilation of inorganic ammonia N similar to their proposed operation in the leaf of the plant (24). Their maxima of activity occurred 6 days apart in pod ontogenesis; thus the association may not be as important in this organ as elsewhere in the plant. The period of maximum pod GOGAT activity (Fig. 5) corresponds to that reported by others for PEP carboxylase activity in the young pea pod (14). This latter enzyme is involved in the reassimilation of CO_2 respired by the developing pea seed (12, 14), especially in the early life of the fruit (9, 10, 12), Kipps and Boulter (16) found that the bulk of this reassimilated carbon was exported to the bean seed as aspartate. It is known that aspartate can be synthesized from carbon skeletons arising from CO_2 fixed through the activities of PEP carboxylase and from amino N donated from glutamate through the activities of aspartate aminotransferase (13). We suggest that the glutamate required in this system could be supplied through the activities of pod GOGAT metabolizing the glutamine imported from the leaf during the early life of the pod.

GOGAT activity in the developing cotyledons increased during growth and N accumulation in the organs (Fig. 2). This activity corresponds to the time of maximum pod GS activity and to the disappearance of protein and free amino acids from the leaf, then pod (Figs. 2 and 4). It has been suggested that these amino acids are translocated to the ripening ovule as amides (34, 35) and that the function of GOGAT in the cotyledons is to metabolize the glutamine imported from the supply organs (6). There was sufficient *in vitro* activity to accomplish this role in the cotyledons (Table III) and the developmental evidence provided in this paper supports the earlier conclusion.

An extremely low level of GS activity was present in the developing pea cotyledons (Table III). Thus it appears that this limited assimilatory capacity would be insufficient to utilize free ammonia which might be released from imported glutamine or asparagine. The failure to detect glutaminase or asparaginase (ref. 6 and unpublished results) in extracts of pea cotyledons is consistent with this speculation.

Glutamine is an extremely reactive metabolite which lies at the center of cellular N metabolism and donates amide N to numerous substrates. However, the obvious importance of the other amide, asparagine, has not been ignored. Recent studies into the mechanism of action of asparagine synthetase suggest that glutamine and aspartate are the physiological substrates (17, 18, 42, 47). The operation of this glutamine requiring enzyme would add importance to the suggested roles of leaf and pod glutamine synthetase activity. However, glutamine-dependent asparagine synthetase activity has not been demonstrated, to date, in green plant tissue.

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