Changes in Activities of Enzymes of Nitrogen Metabolism in Seedcoats and Cotyledons during Embryo Development in Pea Seeds[1]

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ABSTRACT

In the seedcoats of developing pea seeds, the maximal activities of asparaginase (EC 3.5.1.1) and aspartate: α-ketoglutarate aminotransferase (EC 2.6.1.1) are attained early in development, before the embryo has expanded to fill the embryo sac. These two enzyme activities could account for the early absence of asparagine and aspartate from the fluid secreted by the seedcoats into the embryo sac.

Changes in the activities of alanine: α-ketoglutarate aminotransferase (EC 2.6.1.2), glutamate dehydrogenase (EC 1.4.1.3), glutamine synthetase (EC 6.3.1.2), and glutamate synthase (EC 1.4.1.13) have also been measured, in cotyledons as well as seedcoats. On a fresh weight basis, the highest activities of asparaginase and both aminotransferases developed in the seedcoats, whereas the highest activities of the remaining enzymes developed in the cotyledons.

The data indicate that the amide groups of imported asparagine and glutamine are metabolized differently, largely by asparaginase and glutamate synthase, respectively. The NH_4^+ released by the action of asparaginase is evidently reassimilated in cotyledon cells by the joint action of glutamate dehydrogenase, glutamine synthetase, and glutamate synthase. The data emphasize the central importance of α-ketoglutarate-glutamate cycling in the redistribution of amino groups associated with the net synthesis of amino acids and reserve proteins.

A number of recent studies have attempted to elucidate the nutritive and regulatory influences that the living seedcoats exert upon the embryo during its development (4, 17–19). Lacking the phenolic compounds that hinder the extraction of proteins from the seedcoats of many other species, developing pea seeds (*Pisum sativum* L.) offer an ideal system in which to study changes in the activities of enzymes that may be involved directly in the metabolism of seedcoat tissues and, thus, in the regulation of substrate availability to the embryo. This paper reports the results of an investigation of the changing distribution of activities of key enzymes of N metabolism between seedcoats and embryo during the course of pea seed development.

[2] Abbreviations: GDH, glutamate dehydrogenase; GOGAT, glutamine: oxoglutarate amidotransferase or glutamate synthase; GS, glutamine synthetase; OG, α-ketoglutarate.

MATERIALS AND METHODS

PLANT MATERIAL AND PREPARATION OF EXTRACTS

Plants of tall (cv. Telephone) and dwarf (cv. Melbourne Market) varieties of garden pea, *P. sativum* L., were grown, respectively, in the open garden or in pots of garden soil. Flowers were tagged at full blossom and pods then were removed at intervals throughout development, which occurred in spring (October, November) for cv. Melbourne Market and from November to mid-December (1979) for cv. Telephone.

The procedures for extraction of separated seedcoat and cotyledon samples were as described (17, 18), with the sample size increased to four or five seeds. The extraction medium consisted of 25 mM K^+-phosphate (pH 7.5), 0.5 mM 2-mercaptoethanol and 0.03% (v/v) Triton X-100. After centrifugation (17), duplicate aliquots were removed for treatment with ethanol, for the determination of protein content (ethanol-insoluble) and α-amino group content (ethanol-soluble) as previously described (16, 17).

Measured portions of each clarified extract (1.0–1.5 ml) were passed through columns of Sephadex G-25 (0.9 × 25 cm) equilibrated with extraction medium to obtain enzyme solutions free of NH_4^+ and amino acids. Complete removal of ethanol-soluble, ninhydrin-positive metabolites from the excluded fractions was confirmed by analysis (17).

ENZYME ASSAYS

All but two of the enzyme activities of interest were assayed immediately at 25 C (within 3 h of extraction) as extract-dependent linear rates of decrease in A at 340 nm. The unit of enzyme activity is 1 μmol/min under the stated assay conditions. Details of each reaction mixture (final volume, 1.0 ml) are given in the following sequence adopted for routine analysis.

Glutamate dehydrogenase (EC 1.4.1.3). This reaction mixture consisted of 0.27 mM NADH, 6.25 mM OG^2 (K^+), 50 mM $(NH_4)_2SO_4$, 37.5 mM K^+-phosphate (pH 7.5), and 100 μl extract (5, 25, 28).

Alanine:OG aminotransferase (EC 2.6.1.2). This reaction mixture consisted of 0.27 mM NADH, 6.25 mM OG, 10 mM L-alanine, 2.73 units lactate dehydrogenase (EC 1.1.1.27; Boehringer No. 127 221), 46 mM K^+-phosphate (pH 7.5), and 20 or 50 μl extract (8, 27).

Aspartate:OG aminotransferase (EC 2.6.1.1). The same procedure was used as for Ala:OG aminotransferase, but L-aspartate and 3 units malate dehydrogenase (EC 1.1.1.37; Boehringer No. 127 248) were substituted for L-alanine and lactate dehydrogenase, respectively.

Asparaginase (EC 3.5.1.1). The mixture for the first method

(with cv. Melbourne Market) consisted of 0.27 mM NADH, 6.25 mM OG, 12 mM asparagine, 0.6 units GDH (EC 1.4.1.3; Boehringer No. 127 086); 62.5 mM K^+-phosphate (pH 7.5), and 100 μl extract. For the second profile (cv. Telephone), extract-catalyzed formation of aspartate from asparagine was assayed by substituting asparagine (12 mM) for aspartate in the malate dehydrogenase-linked system used for the Asp:OG aminotransferase assay. This latter activity exceeded asparaginase activity of the same extracts (Figs. 5 and 6).

GOGAT (EC 1.4.1.13). This reaction mixture consisted of 0.13 mM NADH, 6.25 mM OG, 0.5 mM glutamine, 1 mM EDTA, 100 mM Hepes (pH 7.0), 2.5 mM K^+-phosphate (with extract), and 100 μl extract (22).

GS (EC 6.3.1.2) and γ-glutamyl transferase. Samples of each extract (post-Sephadex) were concentrated in a Minicon Macro-solute Concentrator, mixed with glycerol to 50% of final volume, and stored at -20 C. These extracts then were transported from one laboratory (D. R. M.) to another (I. R. K.) for assay according to refs. 22 and 23, respectively. In the latter, Hepes buffer (pH 7.3) was used instead of imidazole. Both activities were shown to be stable under these storage conditions.

All solutions of substrates (Sigma) were prepared in buffer, and their pH values were finally adjusted. All except NADH (prepared fresh daily) were stored at -20 C for periods up to 3 weeks.

RESULTS

Parameters of Growth and Development. The changes in fresh weight, dry matter, protein, and soluble amino N contents that occurred during seed development are shown for the seedcoats and a single cotyledon for each cultivar (Figs. 1 and 2). The more rapid development of the seeds of cv. Telephone (Fig. 2) was the result of hot weather (many days with maxima above 30 C) and longer day lengths.

In both instances (Figs. 1 and 2), the early maximal content of free amino acids in the seedcoats was similar in magnitude to that observed previously for cv. Telephone (17). The maximal amino
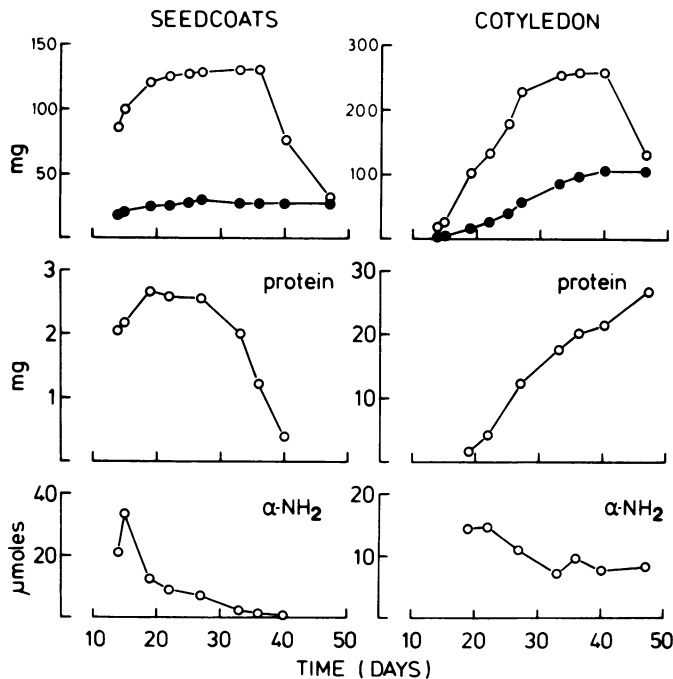
Fig. 2. As for Figure 1, but cv. Telephone.

Fig. 1. Changes in fresh weight (O), dry matter (●), protein, and soluble amino N contents of seedcoats and cotyledons of pea seeds (cv. Melbourne Market) during development. Data are expressed per seed (seedcoats) or per single cotyledon.
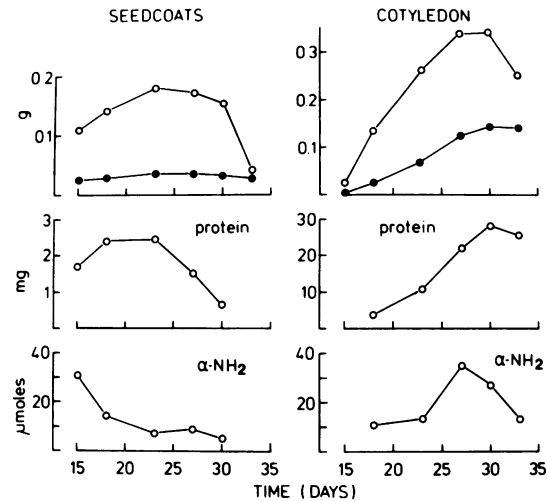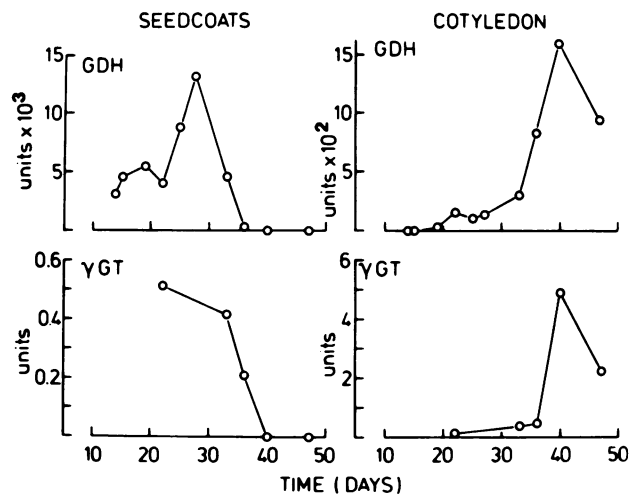
Fig. 3. Changes in the activities of GDH and γ-glutamyl transferase (γ-GT) per plant part for developing seeds of cv. Melbourne Market. Note the scale differences indicated along each ordinate. The unit of enzyme activity is 1 μmol/min at 25 C under the stated assay conditions.

acid content of the cotyledons preceded the onset of rapid net protein synthesis in cv. Melbourne Market (Fig. 1), but not in cv. Telephone (Fig. 2). The final amino acid content of the cotyledons was, in both instances, less than the value previously observed for cv. Telephone (17).

Changes in Activities of Enzymes of NH_4^+ Assimilation. After some fluctuation, GDH activities were highest in the seedcoats at 27 days, relatively late in their development (Figs. 3 and 4). Similar fluctuations were observed in the development of GDH activities in the cotyledons (Figs. 3 and 4), where the values of the maxima differed considerably, according to variety. Table I allows further comparison of the maximal GDH activities from all of these sources on a fresh-weight basis.

The γ-glutamyl transferase activities of the seedcoats reached maximal values at stages preceding their maximal GDH levels (Figs. 3 and 4). The maximal γ-glutamyl transferase activities of the cotyledons were up to 5-fold higher than those of the seedcoats (Table I) and were attained relatively late in development, as for GDH. Measurements of γ-glutamyl transferase activity generally reflect much lower GS activities (13). This was confirmed for both seedcoats and cotyledons, and the maximal GS activities are shown for comparison in Table 1.

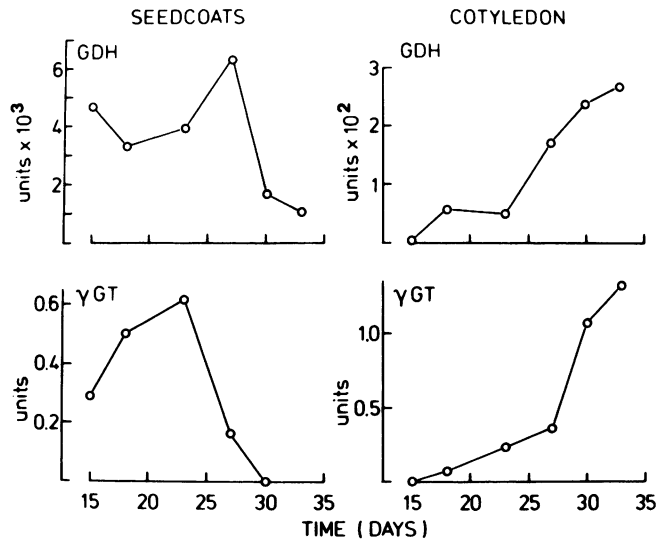**Changes in Activities of Enzymes Catalyzing Transfer of Amide**

FIG. 4. As for Figure 3, but cv. Telephone.

Table I. *Maximal Enzyme Activities of Seedcoats and Cotyledons of Developing Pea Seeds*

Except for 15-day samples, the stage of development is indicated as follows (in days): a, 14; b, 18; c, 22; d, 23; e, 25; f, 27; g, 30; h, 33; i, 36; j, 40; k, 47. MM: cv. Melbourne Market; T: cv. Telephone.

Enzyme	Tissue			
	Seedcoats		Cotyledons	
	MM	T	MM	T
	enzyme units/g fresh wt			
GDH	0.10[f]	0.043	0.71[k]	0.070[g]
γ-Glutamyl transferase	4.11[c]	3.56[b]	19.1[j]	5.34[h]
Glutamine synthetase	0.90[k]	1.03[d]	1.59[k]	0.86[h]
Asparaginase	0.12[a]	0.06	0.049[e]	0.028[d]
Asp:OG aminotransferase	8.31	6.43	2.07[h]	2.22
Ala:OG aminotransferase	3.81[a]	2.32	1.05[k]	1.08[b]
GOGAT	0.10[c]	0.06	0.16[i]	0.13[d]

and Amino Groups. The asparaginase activity of young seedcoat tissues appears to be largely responsible for the generation of free NH_4^+ released into the embryo sac liquid (17). The maximal asparaginase activities of the seedcoats were observed at the earliest stages sampled (Figs. 5 and 6), while liquid still occupied about half the volume of the sac (18). The maximal asparaginase activities of the cotyledons developed relatively early (Figs. 5 and 6). On a fresh-weight basis, these were lower than the seedcoat maxima (Table 1).

Aspartate:OG aminotransferase activities of the seedcoats were also maximal at the earliest stages assayed (Figs. 5 and 6) and had declined substantially by 27 days (Fig. 5) and 22 days (Fig. 6) when the alanine:OG aminotransferase activities were maximal. In the cotyledons, the alanine aminotransferase activity increased to a maximum very early, then fluctuated close to this value throughout development (Figs. 5 and 6). These maxima were similar for both cultivars. In contrast, there was a striking difference in the value of the maximal activity of aspartate aminotransferase in the cotyledons of each cultivar, and also in the time of attainment of maximal activity (Figs. 5 and 6). Despite these differences, the maximal activities of both aminotransferases in the seedcoats clearly exceeded those of the cotyledons on a fresh weight basis (Table I).

In cv. Melbourne Market, GOGAT activity increased in the seedcoats to a maximum at 22 days (Fig. 5). In cv. Telephone, the
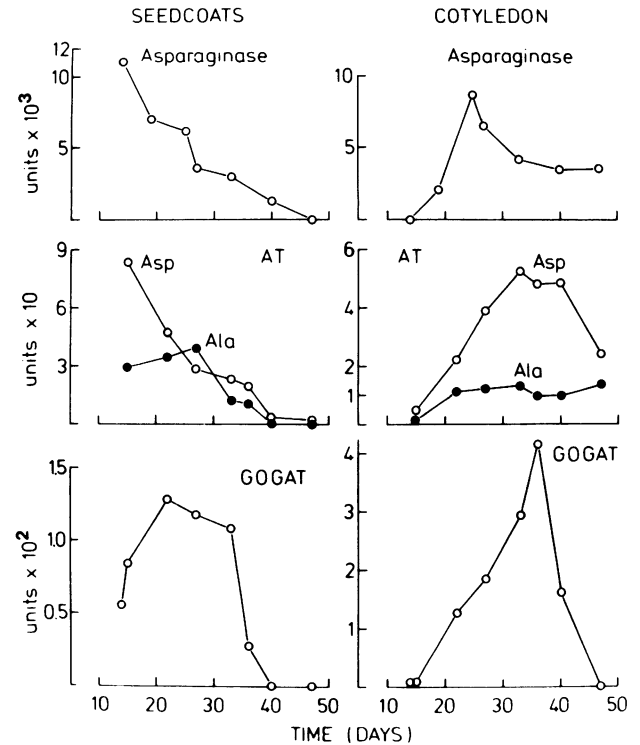

FIG. 5. Changes in the activities of asparaginase, asp:OG aminotransferase (O), ala:OG aminotransferase (●), and GOGAT per plant part for developing seeds of cv. Melbourne Market. Note the scale differences indicated along each ordinate.
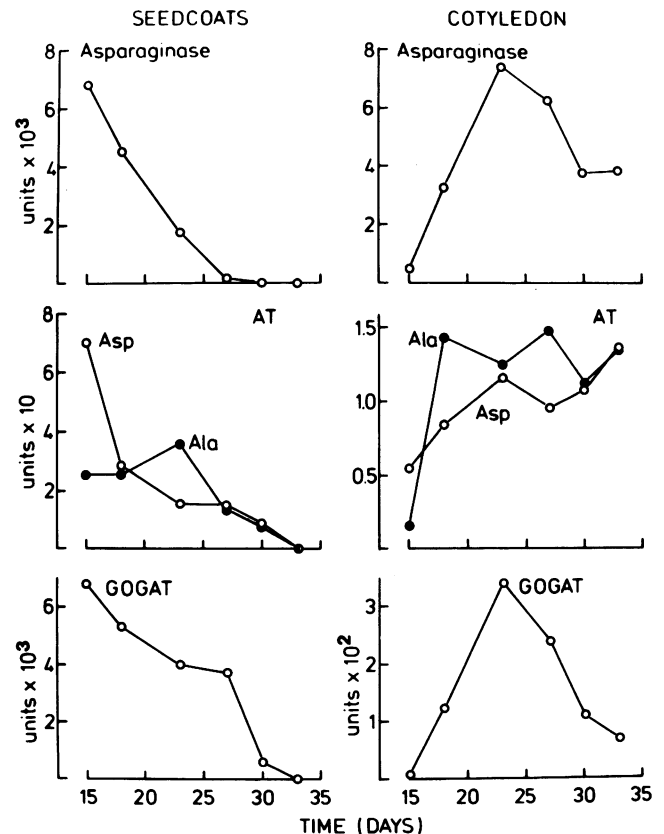

FIG. 6. As for Figure 5, but cv. Telephone.

initial activity was maximal (Fig. 6). GOGAT activity of the cotyledons approached a maximum that was in one instance coincident with the maximal asparaginase activity (cv. Telephone, Fig. 6), but at a later stage in the other (cv. Melbourne Market, Fig. 5). On a fresh weight basis, the maximum GOGAT activity of the cotyledons was about double the maximal activity of the seedcoats (Table I).

DISCUSSION

Transmission of Soluble Nitrogenous Compounds by the Seedcoats. In the earliest stages of postfertilization development, the integuments of the ovule release a nutrient-rich fluid that fills the embryo sac and bathes the expanding embryo asymmetrically (18). A clear distinction may be drawn between this liquid, originating from the integuments, and the endosperm, which is transient and never becomes fully cellular (17, 18). This fluid diminishes in volume as the embryo expands but persists as a sticky coating around the embryo throughout seed development.

One remarkable and puzzling feature of this seedcoat secretion is the very high concentration of ammonia that may be present initially: maximum levels of 100 mM have been reported for kidney bean (24), 70 mM for lupin (1), and 40 to 120 mM according to variety for pea (17, 18). A number of questions arise from these observations: Which enzymes are responsible for the generation of NH_4^+ by the seedcoats? How are they, and their substrates, compartmentalized? How does the developing embryo survive such high external concentrations of NH_4^+? What are the regulatory effects of NH_4^+ at this stage of embryo development?

The present investigation has provided information that perhaps answers the first and third questions. The early maximal activities of asparaginase and aspartate aminotransferase in the seedcoats (Figs. 5 and 6) indicate the mechanism for the selective utilization of aspartate and asparagine from incoming phloem sap (12), to the extent that these two amino acids are very minor constituents of embryo sac liquid (6). The specific activity of asparaginase in seedcoat tissues (Table I) is comparable to the maximum of 0.08 units/g fresh weight measured for lupin cotyledons by Atkins *et al.* (1). The action of asparaginase yields NH_4^+ and aspartate, which together with imported aspartate could provide the C_4 unit for export as a number of C_4-dicarboxylic acids (24). The maximal activities of both aspartate and alanine aminotransferases in seedcoat cells (Table I) are the highest of any tissue of the pea plant (8, 27).

A scheme summarizing the involvement of these key enzymes of amino and amide group transfer in the metabolism of young seedcoats is presented in Figure 7. At this stage, the seedcoats have their maximal content of free amino acids (Figs. 1 and 2), and they are net exporters of homoserine, glutamine, alanine, and certain other amino acids (6), citric acid and C_4-dicarboxylic acids (24), and NH_4^+ (17, 18).

Accumulation of Nitrogenous Reserves in the Cotyledons. Although asparagine is initially lacking in the assortment of amino acids available for uptake by the embryo (6), it has been shown that, as sole source of N, asparagine is able to support limited growth of pea cotyledons cultured *in vitro* (14). The increasing and persistent asparaginase activities of the cotyledons (Figs. 5 and 6) provide evidence of their capacity to utilize this source of amide N *in vivo*. Asparagine is probably increasingly available from the seedcoat secretion as its concentration increases in the seedcoats (6) and as the asparaginase activity of the seedcoats declines (Figs. 5 and 6). The same is true of aspartate (6), which correlates well with declining aspartate aminotransferase activity of the seedcoats (Figs. 5 and 6).

The reassimilation of NH_4^+ generated inside cotyledon cells by the action of asparaginase is evidently brought about by the joint action of GDH, GS, and GOGAT, as discussed below. It is clear that NH_4^+ released inside the cells of the embryo is dealt with
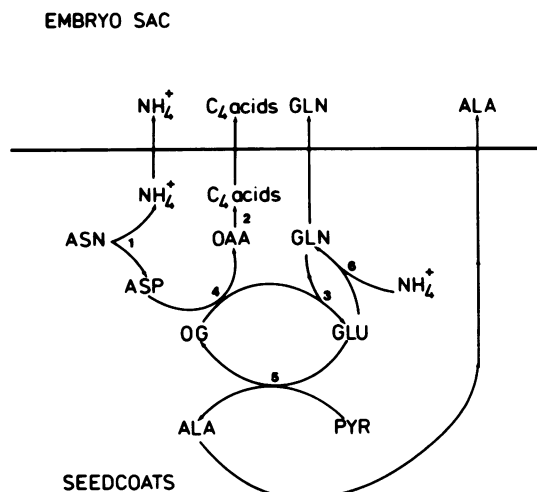

FIG. 7. Amino acid metabolism of seed coats early in pea seed development. 1, Asparaginase; 2, malate dehydrogenase; 3, GOGAT; 4, aspartate aminotransferase; 5, alanine aminotransferase; 6, GS.
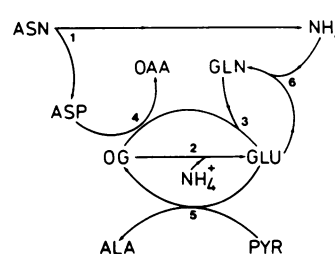

FIG. 8. Amino acid metabolism of cotyledons during pea seed development. Enzyme key as for Figure 7, except 2: GDH.

differently from the exogenous NH_4^+ present in the embryo sac liquid inasmuch as the studies of cultured cotyledons referred to previously (14) showed that external NH_4^+ would not support growth as sole N source. These observations suggest that the peripheral cells of the embryo admit NH_4^+ only at a very slow rate. This conclusion is supported by the present observations on the changes in GDH and GS activities of developing cotyledons (Figs. 3 and 4). These activities are relatively low when the amounts of exogenous NH_4^+ are highest.

Figure 8 presents a scheme showing ways in which imported asparagine, aspartate, glutamine, and glutamate appear to be metabolized in developing cotyledons. A central role for OG-glutamate cycling to mediate redistribution of amino groups to newly synthesized amino acids is indicated. A role for GOGAT in metabolizing imported glutamine in developing pea cotyledons was first proposed by Storey and Beevers (2, 26). These authors, however, concluded that GS activities of developing cotyledons were insufficient to account for the utilization of NH_4^+ derived from the amide group of asparagine or glutamine (26). In Table II, a comparison is made of the maximal mean rates of accumulation of amino N (as protein) with the average daily rates of enzyme activities of developing cotyledons. Taken together, the measured enzyme activities are more than adequate to account for the *in vivo* rates of amino group accumulation.

We have proposed that GS, GOGAT, and GDH act together to reassimilate endogenous NH_4^+ derived from the amide group of asparagine (Fig. 8). Although the action of GDH to form glutamate in plant cells has been discounted recently by some authors (13), the development of GDH activities in cotyledon cells is itself evidence of some function, and amination is the thermodynamically favored direction at physiological pH (5, 25, 28). It has been established that GDH operates as the sole means of

Table II. *Maximal Mean Rates of Accumulation of Amino N as Protein by Developing Cotyledons Compared to Measured Enzyme Activities*

Enzyme	MM, 22 to 27 days[a]	T, 23 to 27 days
	$\mu mol/day \cdot cotyledon$ pair	
Amino N into protein[b]	26.4	44.8
GDH	43.2	31.7
GS	144	115
GOGAT	28.8	83.5
Asparaginase	20.2	19.6
Asp aminotransferase	1,008	305
Ala aminotransferase	173	196

[a] MM: cv. Melbourne Market; T: cv. Telephone.

[b] One mg protein = 8 μmol constituent amino acids.

assimilating NH_4^+ in a number of microorganisms (*e.g.* ref. 3). Evidence for the cooperation of GDH and GS in the assimilation of NH_4^+ has been obtained with labeling studies for the chloroplasts of the siphonous green alga *Caulerpa simpliciuscula* (7) and for nodule tissues for several legumes (9–11). GDH, because of its high K_m for NH_4^+ (5, 25, 28), could effectively counter a build-up of endogenous NH_4^+ through its direct Michaelis rate response in circumstances where GS might be unable to cope with NH_4^+ flux.

The location of GS in chloroplasts from leaves of this species has been established (20, 21). Although we have indicated a possible contribution of GS to the net export of glutamine by the seedcoats (Fig. 7), the GS activities of the seedcoats may well be confined to the chloroplasts in outer cell layers and take no direct part in the net export of glutamine from the innermost parenchyma cells (Fig. 7). In comparison, the GS activities of the cotyledons become greater than those of the seedcoats (Table I) and, since the maximum GS activities occur after the chloroplasts of the hypodermal cells have begun to dissociate, other locations for GS are indicated in the storage cells of the cotyledons.

Further elaboration of the schemes depicted in Figure 7 and 8 may be expected as studies of the compartmentation of enzymes and of their potential substrates are undertaken. The mitochondrion may be expected to feature prominently as a location for some of the enzyme whose total activities were monitored in this study (5, 8, 13, 15).

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