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NITROGEN ECONOMY AND AMIDE METABOLISM DURING SEED DEVELOPMENT IN *PISUM SATIVUM*

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NITROGEN ECONOMY AND AMIDE METABOLISM DURING SEED DEVELOPMENT IN PISUM SATIVUM

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ABSTRACT

The nitrogen nutrition of the developing pea seed is dependent on imported amides provided via the phloem from nearby supply organs. Developing leaves, pods and cotyledons were analyzed for growth, nitrogen content, glutamine synthetase, glutamate synthetase and proteolytic activity to provide correlative information about the mobilization and subsequent utilization of the amides translocated in the fruiting pea (Pisum sativum) plant.

During protein accumulation in the developing cotyledon, the leaf and pod senesced. Loss of protein was associated with an increase in proteolytic activity in the pod, but not the leaf. There was no accumulation of free amino acids during protein depletion in either senescing organ indicating that the breakdown products were immediately exported or metabolized (in situ). Ammonium released from degraded amino acids could have been assimilated into amide by glutamine synthetase. However, the distribution of glutamine synthetase during leaf development indicates that this enzyme may function primarily in the assimilation of inorganic nitrogen. Glutamate synthetase in the developing pod and cotyledon was probably involved in the transfer of amide nitrogen of incoming glutamine to alpha amino nitrogen of amino acids used in reserve protein synthesis. It is proposed that these are key enzymes involved in glutamine metabolism for the supply of nitrogen to developing pea cotyledons.

ACKNOWLEDGEMENTS

I wish to express my gratitude to Dr. Leonard Beevers for his guidance during the course of my graduate research. I would also like to thank Drs. Rice, Cox, Pfiester and Smith for their suggestions and comments concerning this dissertation. I am also indebted to Dr. John Fletcher for his advice and encouagement during my graduate studies.

Special gratitude is expressed to Steve Browder, Larry Hunt, Scott Neely and Jerry Nagahashi for their help, advice, conversation and friendship.

This dissertation is dedicated to my son Justin and my beloved wife Martha, for whom all things matter.

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NITROGEN ECONOMY AND AMIDE METABOLISM DURING SEED DEVELOPMENT IN *PISUM SATIVUM*

CHAPTER I

INTRODUCTION

The protein yield of a legume seed crop depends on the effective operation of those processes whereby the vegetative parts of the plant contribute reduced nitrogen to the ripening fruit. As equally important is the capacity of the developing ovule to utilize this imported nitrogen for storage protein synthesis. Although the species of the translocated nitrogen has been established, very little is known about the nitrogen budget between source and sink organs in the fruiting plant; even less is known about the cascade of enzymes required for mobilization and subsequent utilization of the conserved nitrogen. The purpose of this study was to identify some of these key enzymes and determine their critical role in the cycling of nitrogen during ontogeny of *Pisum sativum* L.

The results of this study are presented in three papers prepared according to the instructions for contributors to Plant Physiology(paper 1 and 3) or Planta(paper 2). The three papers are presented in the same sequence as they were submitted for publication in the two journals.

CHAPTER II

PAPER 1

GLUTAMATE SYNTHETASE IN DEVELOPING COTYLEDONS OF *PISUM SATIVUM*

ABSTRACT

Glutamate synthetase [glutamine (amide): α-ketoglutarate amino transferase oxidoreductase] activity has been demonstrated in the developing cotyledons of Pisum sativum L. c.v. Burpeeana. The enzyme appears to be soluble and is specific for glutamine as amide donor. The enzyme activity is greater with NADH than with NADPH as electron donor.

It is suggested that glutamate synthetase in the developing cotyledon provides a mechanism by which the amide nitrogen of glutamine, from the translocatory stream, is converted into the amino nitrogen of glutamate; transamination reactions involving this synthesized glutamate could provide the amino groups for the biosynthesis of the seed protein amino acids.

INTRODUCTION

The cotyledons of Pisum sativum L. are the sites of deposition of large quantities of proteins. These proteins classically are characterized as the water soluble albumins and the saline soluble globulins. The globulins are considered to be reserve proteins whereas the albumins contain enzymatic proteins (4). Seed development and protein deposition occur concurrently with leaf senescence and it might be expected that the amino acids produced during proteolysis in the senescing leaf could be translocated and serve as the precursors for reserve globulin synthesis in the developing cotyledon. However, the reserve globulins have a different amino acid composition from that normally encountered in proteins from leaf tissue. Lewis and Pate (12) have indicated that the translocatory stream supplying the cotyledons does not contain the complete spectrum of amino acids required for protein synthesis. The translocatory stream is enriched in asparagine and glutamine (16). It has been suggested (12) that the majority of the amino acids required for protein synthesis during cotyledonary development are synthesized (de novo) by utilizing the amide nitrogen.

Lewis (11) has indicated that a pathway which readily makes the amide nitrogen available for amino acid biosynthesis could involve a transamidation reaction to α ketoglutarate via glutamate synthetase [glutamine(amide):α ketoglutarate amino transferase oxidoreductase] to produce glutamate. Transminase reactions involving the synthesized glutamate could provide the amino groups for the biosynthesis of the seed protein amino acids.

The operation of this scheme depends upon the demonstration of the occurrence of the key enzyme glutamate synthetase in cotyledonary tissue. The enzyme has previously been demonstrated in cell tissue cultures (5) root, (7, 10, 17) and leaf tissue and has been implicated in conjunction with glutamine synthetase as providing a principal point of entry of inorganic nitrogen into organic combination (9).

In this communication we report on the occurrence of glutamate synthetase in developing cotyledons of Pisum sativum. The observation is consistent with the speculation of Lewis (11) and it is suggested that activities of the enzyme facilitate metabolism of the amide nitrogen of glutamine into the amino nitrogen of the amino acids utilized in reserve protein biosynthesis.

MATERIALS AND METHODS

Pea seeds (Pisum sativum L. c.v. Burpeeana) were planted in pots containing Vermiculite (Terra-Lite, W. R. Grace and Co., Cambridge, Mass.) and grown in a growth chamber at 3,000 ft—c during a 16 h photoperiod at a day temperature of 23C and a night temperature of 13C. The developing plants were irrigated daily with either a modified Hoagland's solution or distilled water. The plants were staked and labeled fourteen days after germination and flowering dates were recorded daily as reported by Beevers and Poulson (3). Pods were collected twenty-one days after flowering; this is the time of most active protein deposition in the seeds. Seeds were collected from the pod and the testa and embryonic axis removed; the remaining cotyledons were used for enzyme extraction.

Extraction procedures A. Cotyledons were homogenized for 2 min at high speed and 3 min low speed in 50 mM tris-HCl, 1 mM of ethylene diamine tetra acetic acid disodium salt (EDTA), 5 mM 2-mercaptoethanol, pH 7.5 in a Virtis tissue homogenizer at a tissue to buffer ratio of 1:2.5 (w/v). The homogenate was filtered through Miracloth (Calbiochem; La Jolla, California) and centrifuged at 1,000 g for 10 min. The resulting supernatant was centrifuged at 20,000 g for 15 min and the supernatant used for the enzyme assay.

Extraction procedure B. In a modified extraction procedure cotyledons were homogenized with a mortar and pestle in 1:2.5 (w/v) cold buffer solution consisting of 50 mM tris-HCl, 1 mM EDTA, 5 mM 2-mercaptoethanol, 1% bovine

serum albumin and 0.4 M sucrose, pH 7.5. The homogenate was passed through Miracloth and centrifuged at 1,000 g for 10 min. The resulting supernatant was centrifuged at 20,000 g for 15 min and the post 20,000 g supernatant was centrifuged at 100,000 g for 30 min. Aliquots of the 1,000 g, 20,000 g and the 100,000 g supernatants were saved for enzyme assay while the material pelleted during each centrifugation was suspended in the homogenization buffer.

For those enzyme assays in which paper chromatography was used the 100,000 g supernatant was brought to 70% saturation with solid ammonium sulfate and stirred at 4 C for 30 min. The ammonium sulfate precipitable material was recovered by centrifugation at 20,000 g for 15 min. The pellet was suspended in 50 mM tris-HCl, 1 mM EDTA, 5 mM 2-mercaptoethanol, pH 7.5 and then dialysed against 100 vol. of the same buffer for 1 hr at 4 C.

Enzyme assay. Glutamate synthetase activity was usually measured spectrophotometrically at room temperature using a Gilford 240 spectrophotometer and Gilford 6040 recorder. Except where noted, the decrease in absorbance at 340 nm was followed in 3 ml reaction mixtures in which 0.1 ml of enzyme extract (the 100,000 g supernatant from reaction B) was added to 50 mM tris-HCl, 1 mM EDTA, pH 7.5, containing 15 μmoles of α-ketoglutarate and 15 μmoles of L-glutamine, L-asparagine or NH_4 Cl. The reaction was normally initiated by the addition of 250 nmoles of NAD(P)H. The resulting decrease in absorbance at 340 nm was taken to indicate oxidation of reduced pyridine nucleotides. Optimum conditions for the assay were determined and the enzyme activities are expressed as nmoles of NAD(P)H oxidized per minute taken from the slope of the initial linear portion of the reaction curve. Saturation curves were

obtained by varying the concentration of the experimental substrate at fixed saturated concentrations of the other components. Estimates of apparent Michaelis constants (Km) were obtained from Lineweaver-Burk plots of saturation curve data. These plots were linear and regression analysis of the points was performed with the aid of a Hewlett-Packard 9810A Calculator and 9862A Calculator Plotter to determine the slope and intercepts of the least square curve by a poly regression program.

Paper Chromatography

In some assays, in addition to following NAD(P)H oxidation, the formation of glutamate, resulting from glutamate synthetase activity, was detected by paper chromatography. The enzyme reactions, performed as described above, were terminated by the addition of ethanol to an 80% (v/v) final concentration. The resulting precipitated material was removed by centrifugation at 20,000 g for 15 min. One ml of the supernatant was spotted onto Whatman 3 MM chromatography paper and subjected to descending chromatography in the solvent; n-butanol, acetic acid, water (12:3:5). The chromatograms were dried and after being dipped in a cadmium ninhydrin solution (1) were allowed to develop in a darkened glass air tight container containing a beaker of concentrated H_2SO_4.

Protein determination. Protein was precipitated from the enzyme extracts with 5% trichloroacetic acid. The protein pellet recovered by centrifugation at 20,000 g was suspended in 0.1 N NaOH, and the suspension used for the determination of protein by using the Folin phenol procedure of Lowry et al. (13).

RESULTS and DISCUSSION

Enzyme extracts prepared according to procedure A oxidized NAD(P)H in the presence of α-ketoglutarate and L-glutamine (Table 1). However, these extracts also oxidized the reduced pyridine nucleotides in the absence of α-ketoglutarate and glutamine. Thus the activity of glutamate synthetase was calculated from the differences in the rate of NAD(P)H oxidation in the presence and absence of glutamine and α-ketoglutarate(Table 1).

The oxidation of NADH in the absence of glutamine and α-ketoglutarate has been ascribed to an NADH oxidase (5, 7, 8, 14) but also could be due to the activities of any NADH dependent dehydrogenases present in the crude extract.

In attempts to eliminate the high substrate-independent oxidation of NADH, homogenates were prepared with a pestle and mortar in the presence of a buffered sucrose solution as described in procedure B. The 100,000 g supernatant of such extracts showed strict dependence for the presence of α-ketoglutarate and glutamine for NAD(P)H oxidation and thus glutamate synthetase activity could be related directly to pyridine nucleotide oxidation. Analysis of the 1,000 g, 20,000 g and 100,000 g pellets produced in procedure B indicated that glutamine and α-ketoglutarate independent NADH oxidation was associated with the 1,000 g and 100,000 g pellets. Table(2) The activity in the 1000 g fraction probably represents the enzyme associated with intact cells. The NADH oxidation, independent of α-ketoglutarate and glutamine, in the 100,000 g pellet is indicative of an endoplasmic reticulum associated NADH oxidase. No NADH oxidation in the presence of glutamine or α-ketoglutarate was demonstrated by the 20,000 g pellet(Table 2).

Under optimal conditions, glutamate synthetase activity was found to be directly proportional to increased amounts of enzyme extract added up to 0.6 mg of protein in the reaction mixture (fig. 1). The pH optima for glutamate synthetase was found to be 7.5.

Both NADH and NADPH were oxidized by the plant extracts in the presence of α-ketoglutarate and glutamine (Table 1, Fig. 1, 2, 4). A similar lack of reduced pyridine nucleotide specificity has been observed by Fowler et al. (7) and Dougall (5). In contrast, Meers et al. (14) indicate that glutamate synthetase activity in several bacterial species is NADPH specific while that in Rhizobium bacteroids is NADH dependent (17). Robertson et al. (17), in contrast to the observations of Fowler et al. (7), indicate that the glutamate synthetase activity in extracts from lupin roots is NADH specific. At all concentrations tested the rate of oxidation of NADH was greater than that of NADPH. (Fig. 2). Lineweaver-Burk plots of these values were linear over a range 0-200 nmoles NAD(P)H and yielded apparent Michaelis Constants (Km) of 13.3 μM for NADH and 27.7 μM for NADPH. It is possible that the functioning of both NADH and NADPH as electron donors, observed in the present study, represents a lack of pyridine nucleotide specificity of the glutamate synthetase as suggested by Fowler et al. (7) and Dougall (5). However, it is also possible that the activity of a pyridine nucleotide phosphatase could be converting NADPH to NADH (6, 18) which may be the preferred electron donor. The lower efficiency of NADPH in supporting the glutamate synthetase activity favors this latter alternative. Saturation curves for L-glutamine, α-ketoglutarate and NH_4Cl are shown in Fig. 3. Lineweaver-Burk plots of these values were linear and yielded estimates of apparent Michaelis constants of 24.4 mM for NH_4, 1.43 mM for L-glutamine and 0.96 mM for

α -ketoglutarate. No glutamate synthetase activity was found at any of the concentration of L-asparagine tested in the presence of 15 μmoles of α -ketoglutarate.

Asparagine and glutamine occur in the translocatory stream (2, 12, 16) and Millerd et al. (15) have indicated that asparagine is a more efficient nitrogen source than glutamine for the production of proteins and nucleic acids in the cultured cotyledons of Pisum sativum L. These observations imply that the amide group of asparagine is an effective nitrogen source for synthesis of nitrogenous components in developing cotyledons. Fowler et al. (7) originally indicated that asparagine was an effective amide donor for glutamate synthetase in extracts from pea roots. Such a reaction would provide a mechanism for the utilization of the amide group of asparagine in the synthesis of other nitrogenous components. However Lea and Miflin (10) have recently indicated that the findings of Fowler et al. (7) should be questioned since commercially available asparagine is frequently contaminated with aspartic acid which can give erroneous results in glutamate synthetase assays. The glutamine synthetase from the developing pea cotyledon specifically utilized glutamine and other mechanisms must be invoked to account for the metabolism of the amide group of asparagine. The current findings are consistent with the recent report of Atkins et al. (2) which failed to demonstrate glutamate synthetase activity, in extracts of white lupin (Lupinus alba L.), when asparagine was the amide donor. Further confirmation of the inactivity of asparagine in the glutamate synthetase reaction is demonstrated by the inability to detect glutamate formation in the reaction products when asparagine replaced glutamine as the amide donor. (Fig. 5-G.)

The oxidation of NADPH in the presence of α-ketoglutarate and L-glutamine remained linear for at least 5 min(Fig. 4)when 0.1 ml of freshly prepared extract (extraction procedure B) was used. However, routinely assays were conducted for only 3 min. The short incubation times were dictated by the fact that, during prolonged storage (1-2 h) of the enzyme extracts in ice, protein slowly and progressively precipitated from solution. It is believed that this cryo-precipitating protein is a reserve protein component present in the pea cotyledon and its precipitation interfered with the spectrophotometric detection of glutamate synthetase. The precipitate could be removed by centrifugation at 20,000 g for 15 min with no detectable loss of glutamate synthetase activity. However, after a subsequent period of storage on ice, precipitation resumed, necessitating further centrifugation. Oxidation of NADH in the presence of α-ketoglutarate by the plant extract was stimulated slightly by high concentrations of ammonium ions. (Fig. 3.) Thus the extracts apparently contained some glutamic acid dehydrogenase activity. It is possible that a glutaminase was converting the supplied glutamine to ammonia and glutamic acid; the released ammonia could then bring about the oxidation of NADH in a glutamic acid dehydrogenase catalysed reaction. However, that the NADH oxidation measured in the presence of glutamine was attributable to glutamate synthetase and not to the coupled activities of glutaminase and glutamic dehydrogenase is indicated by the observation that glutamine was much more efficient than ammonium in stimulating the oxidation of NADH. Moreover, no glutamate was produced when the enzyme extracts were incubated with glutamine in the absence of α-ketoglutarate (Fig. 5H) thus demonstrating the absence of glutaminase activity.

Although the glutamine stimulated oxidation of NADH in the presence of α-ketoglutarate and plant extracts is indicative of glutamate synthetase activity, confirmation of the enzymatic activity requires the demonstration of the production of glutamate during the reaction. This evidence was provided by paper chromatography of the reaction products from assays which utilized partially purified enzyme extracts. The partial purification of the enzyme removed endogenous amino acids (Fig. 5F) which would have interfered with the detection of the products of the glutamate synthetase reaction. By using the dialysed suspension of the 70% ammonium sulfate precipitable material from the 100,000 g supernatant it was possible to demonstrate glutamine and α-ketoglutarate dependent oxidation of NADH. Paper chromatography demonstrated the progressive production of glutamate during the course of the reaction (Fig. 5A, B, C). No detectable glutamate was produced when α-ketoglutarate and ammonium chloride (Fig. 5E) or α-ketoglutarate and L-asparagine (Fig. 5G) or glutamine (5H) were incubated under similar reaction conditions.

CONCLUSIONS

The developing pea cotyledons contain a glutamate synthetase which shows specificity for glutamine as the amide donor. Both NADPH and NADH serve as reductant in the reaction. However, it is possible that NADPH is converted by a pyridine nucleotide phosphatase to NADH and thus the glutamate synthetase from the pea cotyledon may show a pyridine nucleotide specificity similar to that reported in lupin roots (17).

The presence of glutamate synthetase in the developing cotyledons provides a mechanism whereby the amide group of the glutamine translocated into the cotyledon can be metabolized into the amino nitrogen of glutamate. This glutamate, via transmination reactions, could furnish the amino nitrogen which is utilized in the biosynthesis of the amino acids required for the production of the reserve proteins in the developing cotyledons.

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TABLE 1. The effect of extraction procedure on substrate dependence of glutamate synthetase activity from pea cotyledons. Procedures are described in materials and methods. Complete reaction mixture is 0.2 ml of enzyme extract added to 50 mM tris-HCl, 1 mM EDTA, pH 7.5, containing 15 μmoles of ∝-keto-glutarate, 15 μmoles of L-glutamine was replaced by 15 μmoles L-asparagine or 15 μmoles NH_4Cl as indicated.

REACTION MIXTURE	ACTIVITY			
	Extraction Procedure A		Extraction Procedure B	
	NADH	NADPH	NADH	NADPH
	nmoles oxidized / min.		nmoles oxidized / min.	
Complete	62	42	22	11
Complete — ∝KG	37	29	0	0
Complete — Gln	37	30	0	0
Complete — ∝KG, -Gln	37	30	0	0
Complete — Gln, +Asn	37	29	0	0
Complete — Gln, $+NH_4^+$	44	33	4	3
*Glutamate synthetase	25	12	22	11

* Estimated by subtracting complete — ∝ KG, -Gln from complete.

TABLE 2. Distribution of glutamate synthetase and NADH oxidase activity in cell fractions from pea cotyledons. Fractionation by procedure B as described in materials and methods. Glutamate synthetase reaction mixture of 0.1 ml enzyme extract added to 50 mM tris-HCl, 1 mM EDTA, pH 7.5, containing 15 µmoles L-glutamaine, 15 µmoles ∝-ketoglutarate and 250 nmoles NADH. NADH oxidase assay was performed in the same reaction mixture minus L-glutamine and ∝ketoglutarate. Specific Activity = nmoles NADH oxidized/min/mg protein; Total Activity = nmoles NADH oxidized/m.

ENZYME	ACTIVITY PER CELL FRACTION						
	Crude homogenate	1000 g pellet	1000 g supernatant	20,000 g pellet	20,000 g supernatant	100,000 g pellet	100,000 g supernatant
Glutamate Synthetase							
Specific Activity	43.5	9.8	48.6	2.6	50.6	12.6	54.0
Total Activity	5722.1	182.0	4280.4	92.0	3997.9	68.0	3827.2
Per Cent Yield	100.0	3.2	74.8	1.6	69.8	1.1	66.0
NADH Oxidase							
Specific Activity	78.3	48.4	48.3	0	37.9	68.3	0
Total Activity	10,296.5	900.0	5356.3	0	2878.4	368.9	0
Per Cent Yield	100.0	8.74	52.0	0	27.9	3.6	0

LEGENDS FOR FIGURES

Fig. 1. The influence of enzyme level on the oxidation of NADH (O—O) or NADPH (Δ—— Δ) by glutamate synthetase from pea cotyledons. Assay are as described in materials and methods.

Fig. 2. Saturation curves of NADH and NADPH. Glutamate synthetase was assayed as described in materials and methods but at the indicated concentrations of the reduced pyridine nucleotides. NADH (O—O) NADPH Δ—— Δ

Fig. 3. The influence of alteration of the concentration of component substrates on the rate of oxidation of NADH by glutamate synthetase □—— □ α-ketoglutarate, Δ—— Δ L-glutamine, O——O NH_4^+. No NADH oxidation was observed when L-asparagine replaced L-glutamine. Assays were as described in materials and methods.

Fig. 4. The influence of various nitrogen donors. L-glutamine, NH_4^+ and asparagine on the time course oxidation of NADH (O——O) or NADPH(Δ—— Δ)by glutamate synthetase in extracts from pea cotyledons. Assays were as described in materials and methods.

Fig. 5. Paper chromatography of the amino acids in the reaction products of assays for glutamate synthetase activity. Assays were made by using a partially purified enzyme preparation as described in materials and methods. The complete system is described in materials and methods. A. Complete system, 0 time; B. Complete system, 10 minute incubation. C. Complete system, 20 minute incubation; D. Complete system —L-glutamine, α-ketoglutarate; E. Complete system —— L-glutamine, + NH_4Cl; F. Enzyme extract only; G. Complete system —— L-glutamine, + L-asparagine; H. Complete system — α-ketoglutarate; I. glutamine standard; J. glutamate standard; K. asparagine standard.

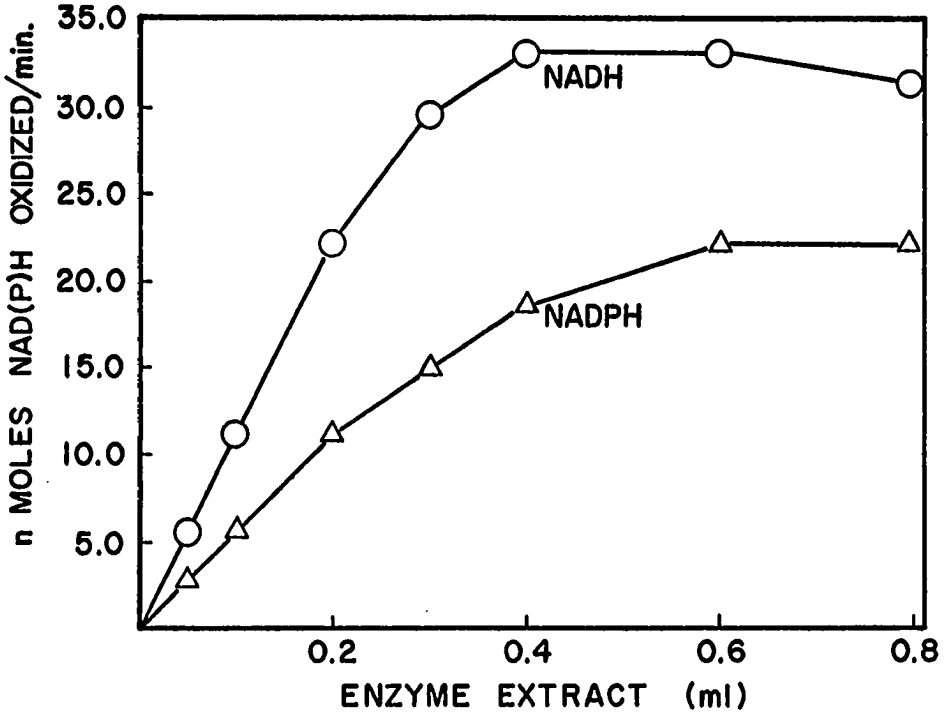
35.0
30.0
25.0
20.0
15.0
10.0
5.0
n MOLES NAD(P)H OXIDIZED/min.
NADH
NADPH
0.2
0.4
0.6
0.8
ENZYME EXTRACT (ml)

FIGURE 1

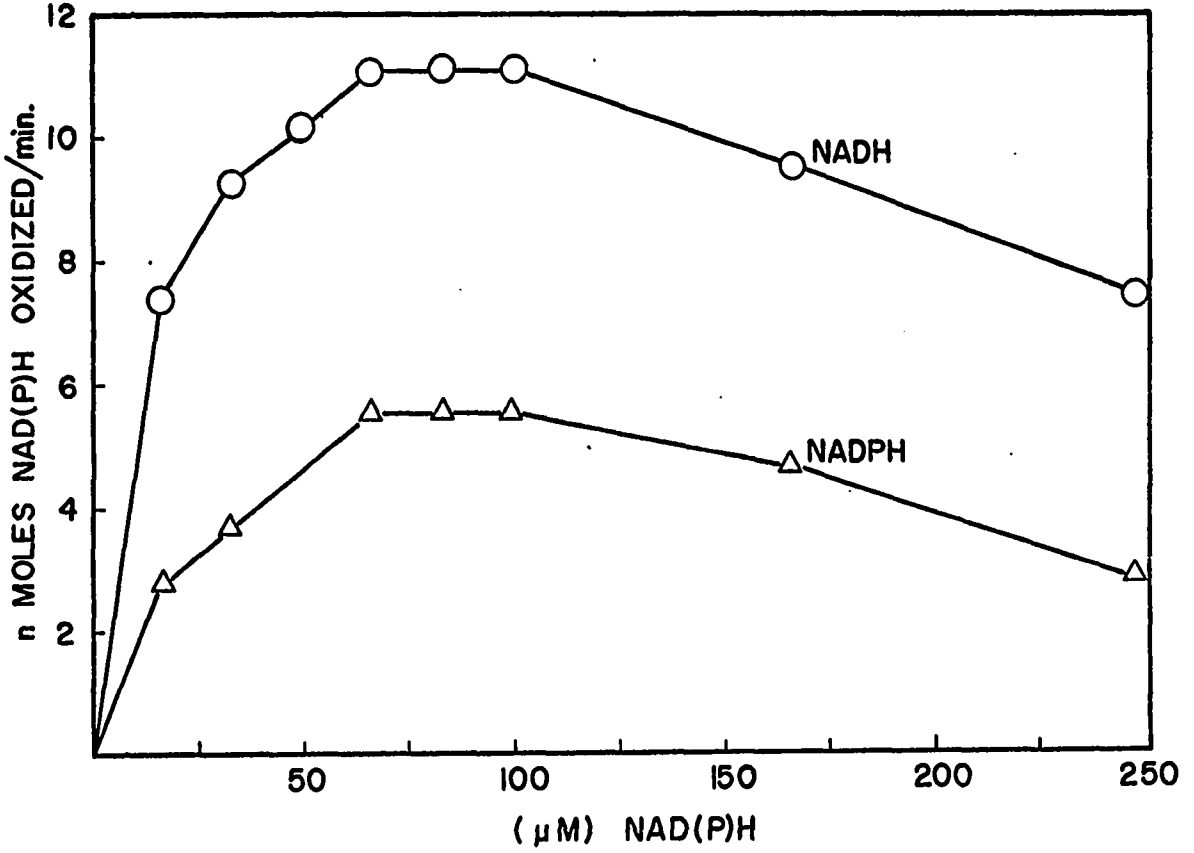
12
10
8
6
4
2
n MOLES NAD(P)H OXIDIZED/min.
NADH
NADPH
50
100
150
200
250
(μM) NAD(P)H

FIGURE 2

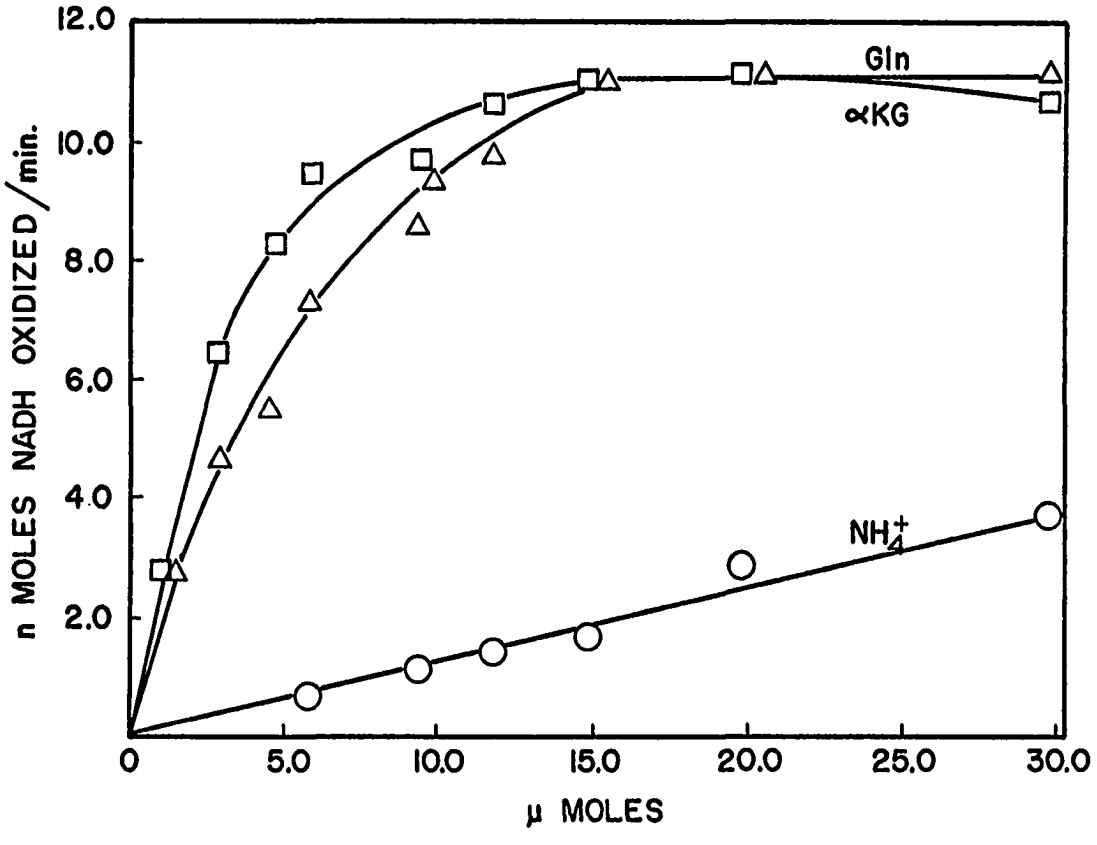
12.0
10.0
8.0
6.0
4.0
2.0
n MOLES NADH OXIDIZED/min.
0
5.0
10.0
15.0
20.0
25.0
30.0
μ MOLES
Gln
αKG
NH4+

FIGURE 3

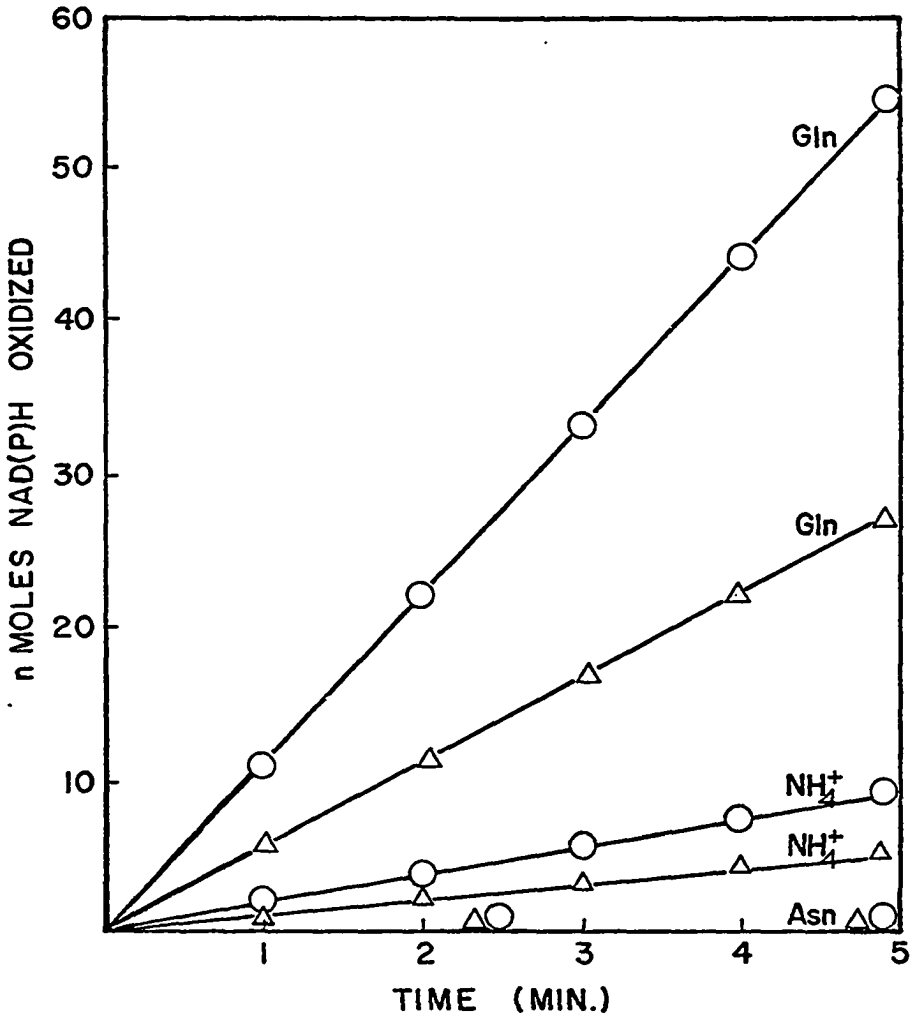
60
50
40
30
20
10
n MOLES NAD(P)H OXIDIZED
Gln
Gln
NH_4^+
NH_4^+
Asn
1
2
3
4
5
TIME (MIN.)

FIGURE 4

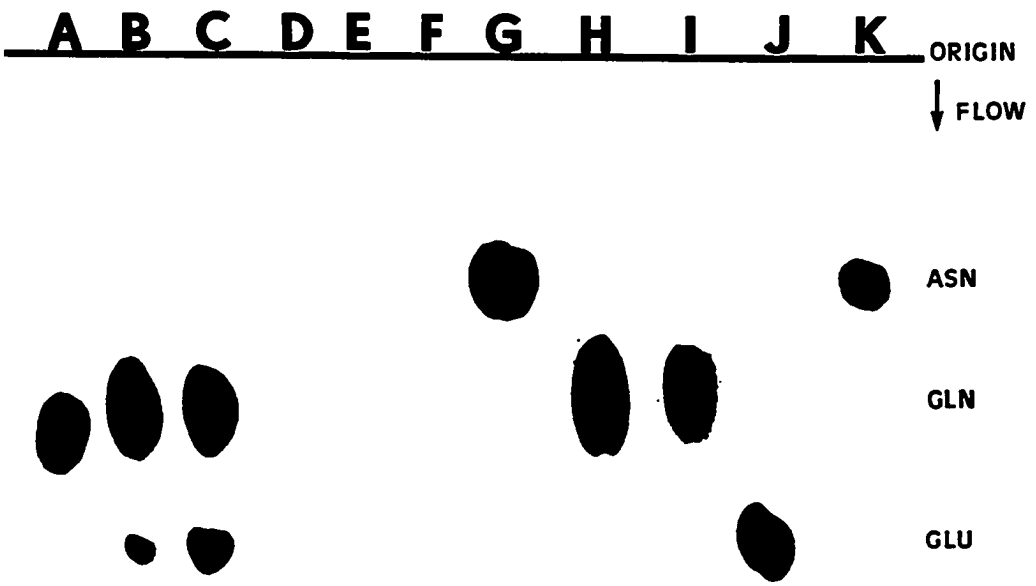

FIGURE 5

CHAPTER III

PAPER 2

PROTEOLYTIC ACTIVITY IN RELATIONSHIP TO SENESCENCE AND COTYLEDONARY DEVELOPMENT IN *PISUM SATIVUM* L.

ABSTRACT

Changes in the weight, chlorophyll, free amino acid and protein content of developing and senescing, vegetative and reproductive organs, of Pisum sativum L. cv Burpeeana were measured. Proteolytic activity in the senescing leaf and subtended pod was followed in relation to these changes.

Protein content decreased in the ageing leaf and pod while the level increased in the developing cotyledon. There was no increase in leaf proteolytic activity associated with a decrease in leaf protein content. In the subtended pod, proteolytic activity increased while protein levels decreased. Estimated (in vitro) proteolytic activity in each ageing organ was greater than actual (in vivo) rates of protein loss.

Proteolytic activity was greater on leaf or pod protein than on non-physiological substrates. Catalysis was stimulated by 2-mercapto-ethanol and ethylenediaminetetraacetate (EDTA). Optimal activity was at pH 5.0. Proteolytic activity was not influenced by addition of ATP to the reaction.

Free amino acids did not accumulate when protein was degraded in senescing supply organs. It is suggested that these amino acids were quickly metabolized in situ or translocated to sink areas in the plant; especially the developing seeds.

Key words: development, leaf, Pisum sativum, pod, protease, senescence.

INTRODUCTION

Developing pea cotyledons are the site of deposition of large amounts of proteins. These proteins are synthesized over a relatively short period of time (Beevers and Poulson, 1972; Basha and Beevers, 1976; Flinn and Pate, 1968; Scharpe and van Parijs, 1973; Millerd, 1975) thus creating a demand for a rapid supply of amino acids. In many varieties of peas, the onset of the development of reproductive structures triggers senescence and it appears that the materials from the nearby vegetative parts are mobilized to support the development of the seed. In particular the leaf and stipule in the reproductive node are important purveyors of nutrients to subtended ripening fruits (Pate et al., 1974; Davies, 1977; Lewis and Pate, 1973; Lewis, 1975; Flinn and Pate, 1970; Lewis et al., 1970; Pate, 1968; Linck and Sudia, 1962; Hopkinson, 1966). Breakdown of proteins during senescence of the leaf could produce amino acids for translocation to the developing seed. The observations that amino acid levels increase in the phloem during leaf senescence are consistent with this concept (Mittler, 1958; Pate et al., 1974 Van Die et al. 1975; Hall and Baker, 1972). In addition to the leaf, the pod is committed to the nourishment of the enclosed seeds (Pate, 1975; Flinn and Pate, 1970; McKee, 1955; Lovell and Lovell, 1970; Muntz et al., 1976; Kipps and Boulter, 1974). Raache (1957) indicated that the nitrogenous compounds translocated from leaves could be assimilated into pod proteins which could then serve as a nitrogen supply for the enclosed seed.

It is evident that the protein of the supply organ must be degraded to provide nitrogenous components to be translocated to the developing

seeds. The initial degradation of the protein must involve hydrolysis to the constituent amino acids. Although there are many reports of proteolytic enzymes in plants (Ryan, 1973), their potential role in providing assimilates for seed development has not usually been considered. However, Peterson and Huffaker (1975), Dalling et al. (1976) and Feller et al. (1977) have investigated the role of leaf proteases in the nitrogen economy of developing cereals and have established a close relationship between the depletion of leaf protein and build up of certain proteolytic activities.

In the present investigation, the proteolytic activity in leaves and pods has been followed during seed development in Pisum sativum. An assessment has been made of the importance of the enzyme(s) in supplying precursors for protein synthesis in the developing cotyledon.

MATERIALS AND METHODS

Plant Material. Pea plants were grown and individual flower anthesis was recorded as described previously (Beevers and Storey, 1976). For all data presented in this paper, the developmental age of an individual organ is quoted as days post anthesis; where anthesis was the state of full bloom of the corolla of the first flower, which usually occurred 30 days after planting.

Studies were confined to the fruit and subtending leaf plus stipule from the first fruiting node of each individual plant. Fruits were harvested and dissected into pod (carpel wall) and ovules. Up to day 9, whole ovules were analyzed, thereafter only cotyledons were utilized. Leaflets (ugually 4) were removed from the rachis of the compound leaf and combined with the large stipules in the same fruiting node. This combination was analyzed together and is designated the leaf in this paper.

Physical and Biochemical Measurements. To determine fresh weight and dry weight, individual parts from 30 to 40 separate plants were harvested, weighed immediately and then again after drying for 30 hr in an 80° oven. For biochemical analysis, freshly harvested tissue was extracted in cold 200 mM sodium-phosphate buffer (pH 7.5) containing 0.3 M NaCl at a ratio of 4 ml buffer per gram tissue. The mixture was macerated in a Polytron homogenizer (Model PT-10-35, Brinkman Inst., N.J.) for 30 sec at medium (setting 6-7) speed. Some tissue from mature pods, leaves or cotyledons required the addition of a further 2 to 4 ml buffer

during homogenization. The homogenate was filtered through miracloth (Calbiochem), brought up to 80% ethanol (v/v) by addition of 95% ethanol and processed for chlorophyll, alpha amino nitrogen and protein estimation as described by Beevers (1974). Chlorophyll was determined by the method of Arnon (1949) in 80% acetone. Protein was determined by the method of Lowry et al. (1951) using Bovine Serum Albumin (Fraction V Sigma Chemical Co., St. Louis, Missouri), as standard. Free alpha amino nitrogen was measured by the ninhydrin method of Yemm and Cocking (1955) using L-leucine as standard. Results for these, and subsequent ninhydrin assays, are reported as µg alpha amino nitrogen (calculated from µg of leucine equivalents). Values quoted for protein, chlorophyll and alpha amino nitrogen content are the mean of triplicate determinations on at least six separately prepared samples from each developmental age of the individual organ.

Extraction of proteolytic activity. All steps were carried out at 5° or less. Freshly harvested, chilled pod or leaf tissue (1 g) was added to 4 ml ice cold 200 mM sodium phosphate buffer (pH 7.5) containing 2mM EDTA, 10 mM 2-mercaptoethanol and 0.1% BSA. The mixture was homogenized (15 sec) in a Polytron homogenizer, filtered through miracloth and centrifuged at 1000 g (2 min) to remove starch and intact cells. The 1000 g supernatant was decanted, then centrifuged at 8000 g for 10 min. The resulting supernatant, which contained all of the detectable proteolytic activity, was applied to the top of a Pharmacia K15 column (Pharmacia Fine Chemicals; Uppsala, Sweden) packed with Sephadex G-50 beads (particle size 50-150µ) to a height of 20 cm. The gel had been swollen and equilibrated in 20mM sodium phosphate-

citrate buffer (pH 7.0) containing 1 mM EDTA and 10 mM 2-mercaptoethanol. The column was eluted by descending flow of equilibration buffer at 3 $ml \cdot min^{-1}$. The effluent was monitored at 280 nm and collected in 3 ml fractions. Protein rich fractions, which were eluted with the column void volume, were pooled, analyzed for soluble alpha amino nitrogen contamination (Yemm and Cocking, 1955) and assayed immediately for proteolytic activity. Similar analysis was done with post void volume fractions. Protein value of the pooled fractions was determined by the method of Lowry et al. (1951).

Preparation of Substrate. Proteolytic activity was assayed with a protein substrate extracted from either pea leaf or pod tissue. The substrate protein was prepared by adding leaf or pod tissue to an extraction medium (1:6; w/v) and homogenizing for 5 min in a Waring Blender. The extraction medium was modified from Sakaro and Wildman (1972) and consisted of 100 mM Tris-HCl (pH 8.3), 1.0 M NaCl, 0.1% sodium dodecyl sulfate (SDS), 5 mM 2-mercaptoethanol and 1 mM EDTA. The homogenate was filtered through miracloth and starch and remaining cellular debris were removed by centrifugation at 1000 g for 2 min. The resulting 1000 g supernatant was brought to 80% ethanol (v/v), stirred in the cold (5°) for 1 hr and placed in the freezer (-12°) overnight. The cold ethanol mixture was centrifuged at 10,000 g for 15 min and the resulting dark green supernatant was discarded. The 10,000 g pellet was washed successively with 95% ethanol, 100% ethanol, ethanol-ether (3:1, v/v) and 5% trichloroacetic acid. The pellet was recovered from each washing by centrifugation. The final acid insoluble material was dissolved in a solution of 0.5 N NaOH, 0.5 M NaCl and 0.01%

SDS, then dialyzed for 72 hr against 3 changes (100 volumes each) of 0.01 N NaOH containing 0.01 M NaCl and 0.001% SDS. The protein rich dialysate was recovered and stored at -12° until needed. Before use in the assays for proteolytic activity, protein substrate was diluted to a concentration of 4 mg protein · ml^{-1} with 100 mM sodium phosphate-citrate buffer, pH 5.0. Other substrates [casein, (National Biochemicals); bacto-hemoglobin, (Difco Laboratories); BSA Fraction V, (Sigma)] used in protease assays were similarly extracted, dialyzed and diluted.

Assay of Proteolytic Activity. Proteolytic activity was detected essentially by the method of Beevers (1968). The standard reaction mixture contained 1.0 ml of protein substrate (see above), 1.0 ml of enzyme extract and 1.0 ml of 100 mM sodium phosphate-citrate buffer (pH 5.0) containing 10 mM 2-mercaptoethanol. Unless otherwise indicated the mixture was incubated at 30°, pH 5.0 for 0 (control) or 60 min. To ensure measurement of catalytic activity under optimum conditions, assays were also conducted over different time intervals and with varying amounts of enzyme of substrate added to the same 3.0 ml reaction. Catalysis was terminated by addition of 1.0 ml ice cold 20% trichloroacetic acid and the resulting precipitated material was immediately removed by centrifugation.

Normally, 0.1 ml of the clear, trichloroacetic acid soluble supernatant was analyzed for ninhydrin positive components (Yemm and Cocking, 1955). An increase of acid soluble alpha amino nitrogen during the time course of the reaction was taken to indicate proteolytic activity. Incubation mixtures containing only substrate or enzyme extract, or boiled enzyme (100°, 15 min) in the same reaction buffer, were similarly treated and analyzed to serve as

standard controls. Except where indicated, pod or leaf substrate was used to detect pod or leaf proteolytic activity, respectively.

To determine the pH optimum for proteolytic activity, 1 ml of substrate and 1 ml of enzyme extract was incubated with 100 mM phosphate-citrate buffer adjusted to the desired pH as described by Frith et al. (1975). The pH of the initial reaction mixture was determined and initial reaction velocities were measured under otherwise optimum conditions.

For developmental studies of proteolytic activity, triplicate assays were run on each of three separately prepared extracts from individual tissue of each age listed. Rates were obtained from the initial linear portion of time progress curves where reaction velocities were first order with respect to time and amount of enzyme extract in the incubation mixture and zero order with respect to substrate concentrations. Units of proteolytic activity are expressed as μg of alpha amino nitrogen released · hr^{-1} under the standard conditions described above. Specific activity is units · mg^{-1} enzyme extract protein in the reaction mixture.

RESULTS AND DISCUSSION

At anthesis of the bloom at the first flowering node, the subtending leaf was approximately 30% fully expanded. Maximum expansion occurred 9 days later. Leaf fresh weight and dry weight increased up to twelve days post anthesis (Fig. 1); thereafter there was a decline in leaf weight. Accurate measurements of the compound leaf length could not be made because the distal end of the rachis terminated in a branched tendril. The leaf-like stipule in the reproductive node was two to three times longer than any of the four leaflets of the subtended leaf blade.

The pod (carpel wall) gained fresh and dry weight rapidly from day 1 until day 12 and continued to increase in fresh but not dry weight, until day 18 (Fig. 1). Maximum pod length (7.2 cm) was attained 6 days after flowering and by 21 days the enclosed 5 or 6 seeds filled the pod. At 24 days post anthesis, the pod began to wrinkle and desiccate but appreciable dry weight was retained at maturity (33 days).

Although ovule enlargement and maturation began soon after anthesis, developing cotyledons were not easily dissected until 6 days post anthesis. The initial cotyledonary development was slow up to day 9 but afterwards fresh and dry weight increased rapidly to day 27. At this time, fresh weight declined resulting in a drying out of the cotyledons (Fig. 1).

The changes in leaf and pod protein followed a similar pattern to fresh weight. Protein accumulated in the leaf and pod up to days 12 and 15 respectively (Fig. 2) and then declined. During senescence, the leaf lost 10 mg protein, and the pod lost over 20 mg protein, while each developing cotyledon accumulated approximately 13 mg of protein (Fig. 2). Chlorophyll

level reached a peak at day 9 in the leaf and declined thereafter (Fig. 3). Leaflet margins began to turn brown after day 15 and by day 30 leaves were visually devoid of chlorophyll. The chlorophyll level of the pod peaked at day 12 and remained relatively constant until day 21. Each pod contained about one half of the chlorophyll found in the leaf. Although the cotyledons appeared green they contained very little chlorophyll in comparison to the leaves and pods (Fig. 3).

Free amino acids accumulated quickly in the leaf and pod during expansion, but the levels rapidly declined after days 9 and 12 respectively (Fig. 4). It is significant to note that amino acid content of the leaf and pod did not increase during the period of protein depletion in these structures. In the developing cotyledon, free amino acid content also increased during the period of most rapid growth and peaked at day 21 post anthesis.

Characterization of proteolytic activity

The original crude homogenates of leaves and pods contained soluble amino nitrogen which interfered with the accurate determination of protein hydrolysis. Proteolytic activity could be recovered from these homogenates by ethanol, acetone or ammonium sulfate precipitation, followed by dialysis. However, these lengthy procedures did not remove all of the contaminating amino acids from the extract and the resulting enzyme preparations had a somewhat lower activity than those prepared by Sephadex chromatography. Therefore, crude homogenates were routinely filtered through Sephadex G-50 columns to provide rapidly recovered proteolytic activity, free from detectable soluble amino acids.

No proteolytic activity was detected in the 1000 g or 8000 g pellets (see materials and methods) of the extraction procedure.

Release of acid soluble, alpha amino nitrogen from protein substrates was linearly proportional to time and amount of enzyme extract added to the reaction mixture (Fig. 5). Similar linearity was exhibited in assays for proteolytic activity in pod extracts.

Optimum proteolytic activity occurred at pH 5.0 (Fig. 6). At pH 3.75 and above catalysis was linear with time and enzyme concentration in the reaction mix. At pH 3.5 and below, non-enzymatic acid hydrolysis of proteins occurred as the release of free amino acids from control assays (substrate only) was approximately equal to release of the amino acids from experimental assays (substrate plus enzyme extract). No free amino acids were released from control assays at pH 3.75 and above. When enzymatic activity was calculated by subtracting control assay values from experimental assay values and then plotted as a function of pH, a symmetrical curve about a peak pH of 5.0 was generated (Fig. 6). This is similar to the pH optimum reported for various acid proteases in tobacco (Tracy, 1948; Anderson and Rowan, 1965), barley (Visuri et al., 1969) and corn (Feller et al., 1977) leaves. Brady (1961) found a pH optimum of 5.9 to 6.3 for clover leaf protease activity on gelatin substrate. Tracy (1948) and Singh (1962) investigated leaf proteases from a number of plants and found optimal activity at pH 5.0 or 5.4.

Leaf proteins were hydrolyzed more readily than non-physiological substrates (hemoglobin, casein, bovine serum albumin) when incubated with leaf extracts (Table 1). Similar results were obtained with pod extracts

incubated with pod proteins or the non-plant protein substrates. A differential sensitivity of non-physiological substrates to leaf proteases has been reported by Anderson and Rowan (1965), Racusen and Foote (1970) and Brady (1961). Spencer and Spencer (1974) have demonstrated greater plant proteinase activity on plant proteins than on casein. In contrast to the observed specificity of proteolysis demonstrated by proteases from leaf extracts, commercial protease hydrolysed all of the proteins tested with approximately equal efficiency.

It has frequently been observed that anaerobiosis retards senescence (James 1953). Under anaerobic conditions, protein content is sustained, suggesting that proteolysis is an aerobic, energy requiring process. Several investigators have attempted to account for this phenomenon without reaching any concensus (Goldberg and St. John 1976). However, Etlinger and Goldberg (1977) recently reported an ATP stimulated proteinase in extracts from reticulocytes. In investigating this possibility in extracts from leaves of Pisum, no ATP stimulation of proteolysis was observed against denatured leaf proteins.

Slightly higher proteolytic activity (8 to 10%) in leaf and pod extracts, was observed when EDTA was included during the homogenization stage. Similar findings have been reported for other plant acid proteases (Harvey and Oaks, 1974; Frith et al., 1975) and endopeptidases (at pH 5.4) (Feller et al., 1977). Additionally, proteolysis was stimulated (3 fold) by inclusion of sulfhydryl reagents, either in the extraction medium or reaction mixture (Table 2). Proteolytic activity in clover and tobacco leaves was found to be stimulated by reducing agents such as thioglycollate and cysteine (Brady, 1961; Tracy, 1948; Anderson and Rowan, 1965). Feller

et al. (1977) indicated that caseolytic activity at pH 7.5 from senescing corn leaves was inhibited by 2-mercaptoethanol but proteolysis at pH 5.4 was stimulated by the sulfhydryl reagent.

The characteristics of proteolytic activity in pea leaf or pod extracts coincide closely to those attributed to acid, sulfhydryl endopeptidases in plant tissue (Harvey and Oaks, 1974; Feller et al., 1977; Ryan, 1973).

Proteolytic activity during ontogeny

Figure 7 shows the changes in extractable proteolytic activity from the ageing leaf and pod. Activity is expressed as a function of fresh weight and protein to facilitate comparison with reports of other workers. However, in agreement with Feller et al. (1977), it is felt that it is more meaningful in developmental studies to consider data on the basis of a parameter that does not change with age and emphasis in this report is thus placed on results expressed on a per organ basis.

Proteolytic activity, extractable from the subtending leaf, increased slowly from day 6 to day 15. Activity then decreased slowly until day 24 and declined more rapidly thereafter. The change in proteolytic activity during leaf development in peas contrasts markedly with that reported in corn leaves where it was observed that a decline in protein was associated with an increase of caseolytic activity (Feller et al., 1977). Dalling et al. (1976) have shown an increase in protease activity coincident with the senescence of wheat leaves. Peterson and Huffaker (1975) report that the decline in ribulose 1-5 diphosphate carboxylase is associated with an accumulation of protease in the senescing barley

leaf. However, others have reported a lack of relationship between protein depletion and an increase in protease activity during senescence of various dicotyledonous leaves. (Beevers 1968, Anderson and Rowan, 1965, 1968; Woolhouse, 1967).

In contrast to the situation in the leaf, proteolytic activity in the pod increased up to 21 days, and was maintained at a high level until day 27. Thus, in the pod protein degradation (Fig. 2) was correlated with a build up of proteolytic activity and the situation appears to be similar to that reported in the leaves of cereals (Feller et al., 1977; Dalling et al., 1976; Peterson and Huffaker, 1975).

The specific activity of the extracted pod proteolytic enzymes declined at maturity while specific activity in the leaf extracts increased (Fig. 7) Anderson and Rowan (1965) observed a similar increase in specific activity of proteases in tobacco leaf tissue. Therefore it appears that pod proteolytic enzymes may be degraded during maturation, whereas, proteolytic enzymes apparently make up a progressively increasing proportion of the residual protein in the senescing leaf.

Enzyme extracts from two separate stages of leaf or pod development, which contained different levels of proteolytic activity, were mixed and assayed. The resulting activity of the mixture was intermediate to the two extremes (Table 3). These data indicate that the results of the developmental studies were not due to the presence of an extracted inhibitor (or activator) which influenced the results of <u>in vitro</u> assays for proteolytic activity.

CONCLUSIONS

The accumulation of protein in the developing cotyledons occurred contemporaneously with the depletion of protein in the subtending leaf and pod. This observation suggests that mobilization of protein in the supply organs could provide precursors for synthesis in the cotyledon. Measured rates of *in vitro* proteolytic activity exceeded actual rates of leaf protein depletion (*in vivo*) by about 5 fold (or 2.5 fold if a Q_{10} of 2 is utilized to account for assay, and growth chamber, temperature differences). However, it is clear that the combined subtending supply leaf and pod protein (11.5 mg and 26 mg respectively) is insufficient to provide all the necessary components for the synthesis of 156 mg of protein (12 x 13 mg) by all the developing cotyledons in a fruit.

On the basis of time course studies of protein depletion in the leaf and accumulation in the pod, it does not appear that the leaf proteins serve as precursors for synthesis of proteins in the pod.

It is possible that the initial mobilization and translocation of protein breakdown products from the supply leaf need not be associated with protein depletion and senescence. Proteins are in a constant state of turnover (Racusen and Foote, 1962 ; Oaks and Bidwell,1970; Huffaker and Peterson 1974). It has been suggested that amino acids released during protein breakdown might not be re-utilized for synthesis (Bidwell et al. 1964). It is possible that the breakdown products from protein turnover are translocated from the supply leaves to the pod and developing seed. There is evidence to support this hypothesis. Pate et al. (1974) have indicated that phloem loading in the leaf draws upon amino acids released during turnover, and Hopkinson (1966) measured mobilization of nitrogen

from the supply leaf before there was visual evidence of senescence (chlorophyll loss). Pate and Flinn (1973) found that even while accumulating nitrogenous compounds, pea leaves could release nitrogen which had been assimilated in early growth. This release of nitrogen increased during ageing and it was concluded that the mobilization of nitrogenous reserves from the supply organ was a continuous process which gathered momentum as the seed developed. The observed levels of proteolytic activity in the leaf (Fig. 7) are consistent with this developmental strategy.

It appears that during senescence the endogenous leaf proteins may become progressively more accessible to degradation by existing proteolytic enzymes. In contrast, the close relationship between pod protein depletion and pod proteolytic activity suggests that in the developing carpel, protein mobilization may be controlled by the level of proteolytic enzymes. This situation is comparable to that proposed for leaf tissue in cereal crops (Dalling et al., 1976; Feller et al., 1977; Peterson and Huffaker, 1975).

In both the leaf and pod, protein depletion during senescence (Fig. 2) is not associated with an accumulation of free amino acids (Fig. 4). Wallace and Pate (1967) reported a similar situation in ageing Xanthium leaves. This is in marked contrast to the accumulation of free amino acids in detached, senescing leaves (Beevers, 1976). Thus, it appears the products of protein degradation are immediately metabolised or translocated out of senescing supply organs.

Pate et al. (1975) have stated that the attraction, processing and export of solute is an activity of mature leaves second only in importance to photosynthesis. This paper provides evidence for the catalytic potential to release amino acids from supply organ protein. It is recognized that the

operation of an integrated, ontogenetical scheme of nitrogen economy, related to seed nutrition, would require the activity of several additional enzymes. Developmental studies into these problems are proceeding in our laboratory.

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Yemm, E. W., Cocking, E. C.: The determination of amino acids with ninhydrin. Analyst 80, 209-213 (1955).

Table 1. Proteolytic activity on different substrates. Extracts were prepared from 18 day old leaves. Commercial protease (Sigma Chemicals) was assayed for comparison. Substrate concentration was 4 mg · ml^{-1}. Activity is expressed as units · ml^{-1} enzyme extract. Commercial protease was not assayed with BSA.

Substrate	Activity	
	Leaf Extract	"Commercial" Protease
Leaf Protein	11.7	28.9
Pod Protein	9.8	29.9
Hemoglobin	6.6	28.1
Casein	5.1	27.6
BSA	0	-

Table 2. Influence of sulfhydryl groups on leaf proteolytic activity. Extracts were prepared from 15 day old leaves as described in materials and methods except the extraction and elution buffer was ± 10 mM 2-mercaptoethanol (SH). Assays were conducted under standard conditions ± 10 mM 2-mercaptoethanol (SH). Similar results were obtained with extracts from pods. Activity = units · ml^{-1}.

Extraction medium	+SH		-SH	
Reaction mix	+SH	-SH	+SH	-SH
Activity	4.8	4.3	4.1	1.7

Table 3. Mixing experiments. Extracts from respective developmental ages (day 12, 30 for leaf; day 12, 27 for pod) were brought to equal protein concentrations with elution buffer, mixed in the proportion indicated and assayed under standard conditions. The reaction mixture contained 1 ml substrate (4 mg pod or leaf protein), 1 ml mixed enzyme extract and 1 ml 200 mM phosphate-citrate buffer (pH 4.0). Specific activity = units $\cdot$ mg^{-1} protein in enzyme extract.

Leaf			Pod		
Per Cent Extract in Assay			Percent Extract in Assay		
Day 12	Day 30	Specific Activity	Day 12	Day 27	Specific Activity
0	100	14.4	0	100	6.9
25	75	11.1	25	75	5.7
50	50	8.5	50	50	4.8
75	25	5.6	75	25	3.4
100	0	2.5	100	0	2.6

LEGENDS FOR FIGURES

Fig. 1. Developmental changes in fresh and dry weight of the leaf, pod and cotyledon from the first reproductive node of the pea plant.

Fig. 2. Protein content of the ageing leaf, pod and cotyledon from the first reproductive node of the pea plant.

Fig. 3. Chlorophyll content of the leaf, pod and cotyledon from the first reproductive node of the ageing pea plant.

Fig. 4. Changes in solube alpha amino nitrogen (Amino N) content of the developing leaf, pod and cotyledon from the first reproductive node of the pea plant.

Fig. 5. Relationship of proteolytic activity to incubation time and amount of enzyme extract in the reaction mixture. Extracts were prepared from 15 day leaves. Reaction mixtures to determine activity as a function of enzyme extract contained 1 ml of leaf protein substrate (pH 5.0), the indicated amount of enzyme extract and 100 mM phosphate-citrate buffer (pH 5.0) to a final volume of 3.0 ml. One ml of enzyme extract in the same reaction mixture was used in time progress assays. Units = µg alpha amino N released · hr^{+}. Activity = µg alpha amino N released.

Fig. 6. Influence of pH on proteolytic activity. Standard reaction mixtures were incubated in 0.1 M phosphate-citrate buffer adjusted to the indicated pH by varying proportions of 100mM sodium phosphate and 100mM sodium citrate. Similar results were obtained with extracts from 24 day leaves and 15 and 27 day old pods.

Fig. 7. Proteolytic activity as a function of developmental age in the leaf (leaflet + stipule) and pod (carpel wall). Assays were run under standard conditions and reaction mixtures contained 1 ml of substrate (4 mg leaf or pod protein), 1 ml of enzyme extract (leaf of pod) and 1 ml of 100 mM phosphate-citrate buffer, pH 5.0. For each developmental age assayed, proteolytic activity was linear with time and amount of enzyme extract in the reaction mixture. Proteolytic activity is expressed as units (μg alpha amino nitrogen released $\cdot hr^{-1}$). Specific activity is units $\cdot mg^{-1}$ enzyme extract protein.

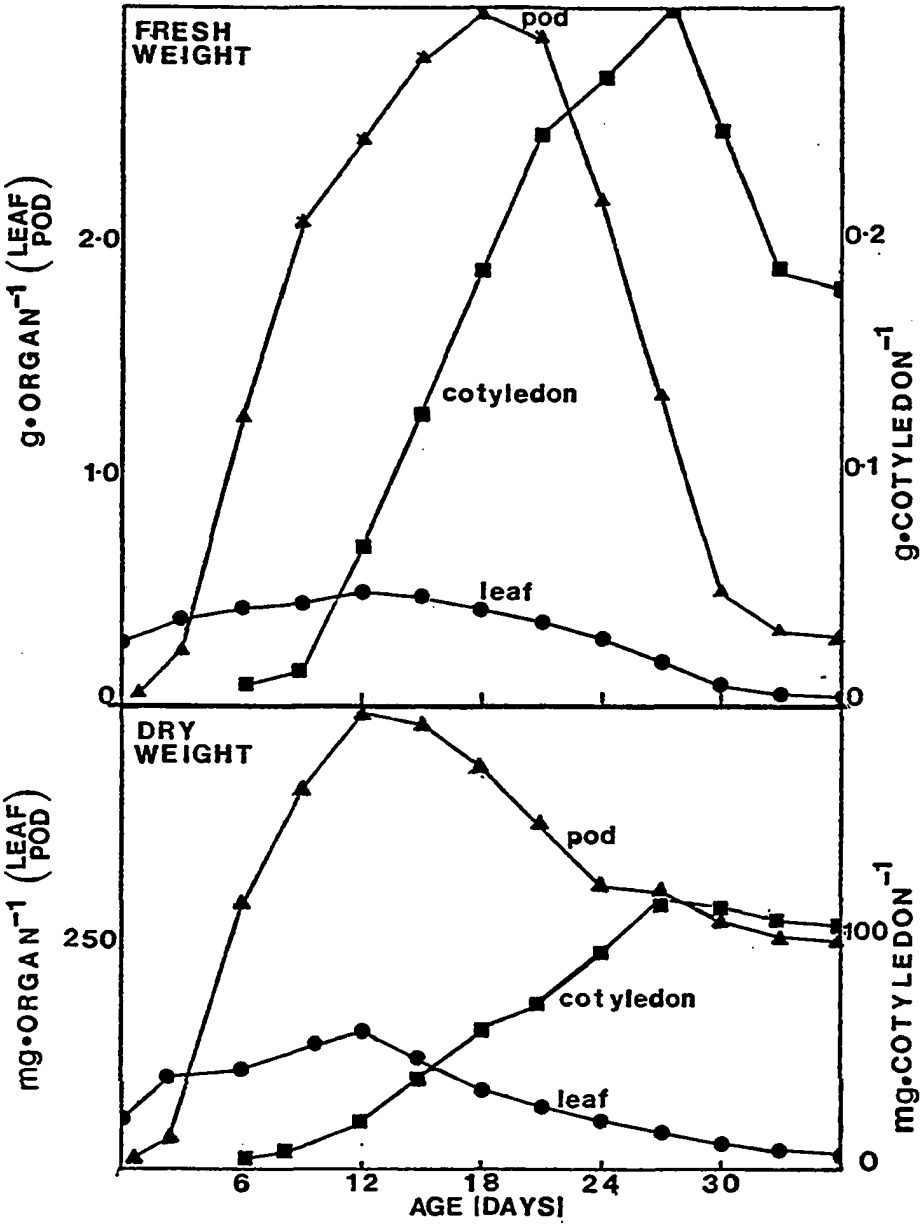
FRESH WEIGHT
pod
cotyledon
leaf
g·ORGAN⁻¹ (LEAF POD)
g·COTYLEDON⁻¹
2·0
1·0
0
0·2
0·1
0
DRY WEIGHT
pod
cotyledon
leaf
mg·ORGAN⁻¹ (LEAF POD)
mg·COTYLEDON⁻¹
250
100
0
6
12
18
24
30
AGE (DAYS)

FIGURE 1

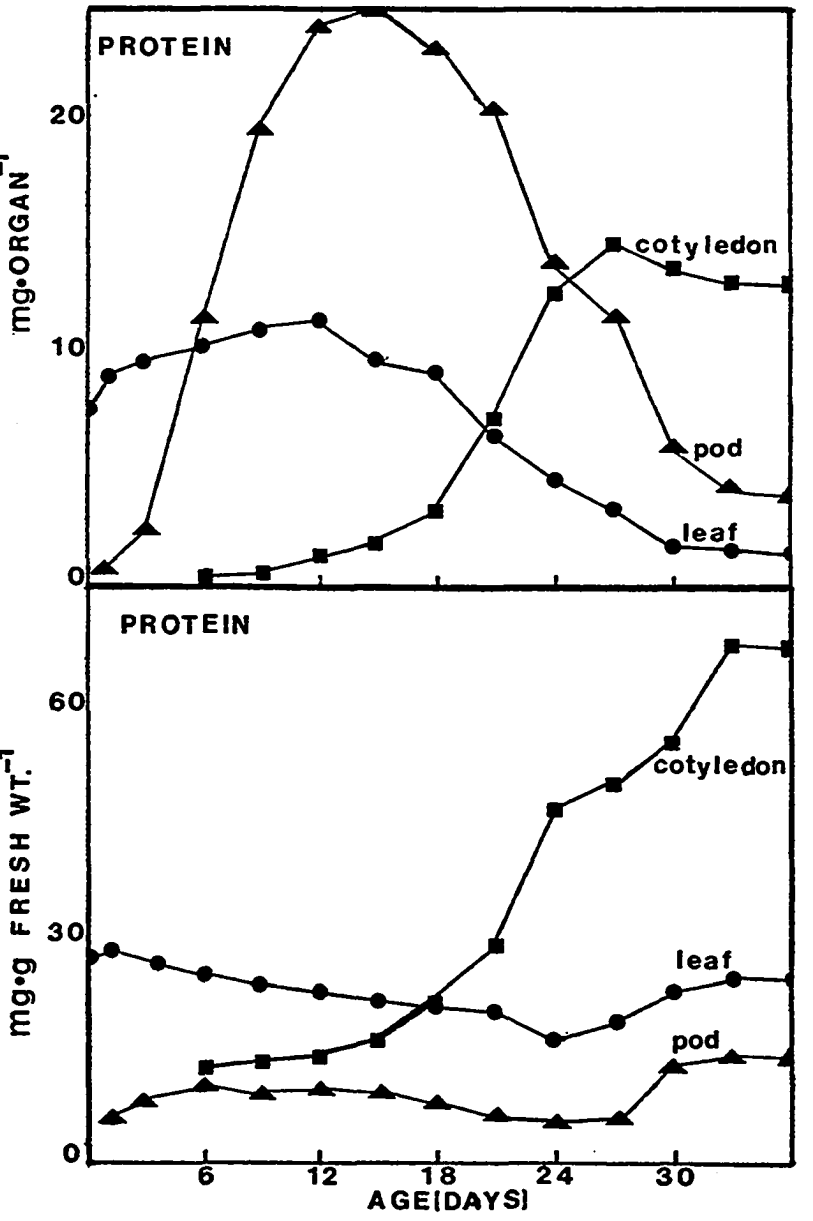
PROTEIN
mg•ORGAN⁻¹
20
10
0
cotyledon
pod
leaf
PROTEIN
mg•g FRESH WT.⁻¹
60
30
0
cotyledon
leaf
pod
6
12
18
24
30
AGE(DAYS)

FIGURE 2

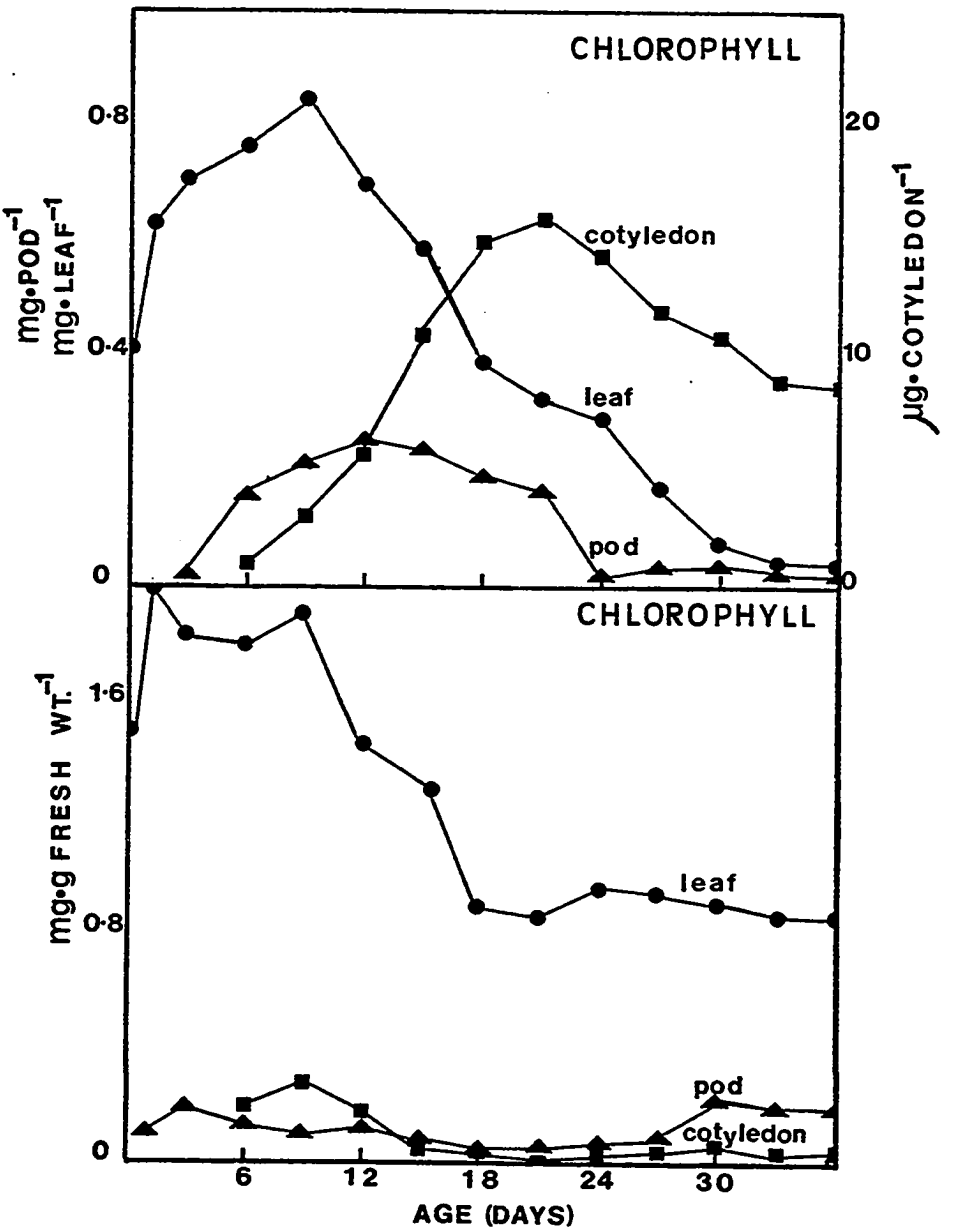
CHLOROPHYLL
0·8
0·4
0
20
10
0
mg·POD⁻¹
mg·LEAF⁻¹
µg·COTYLEDON⁻¹
cotyledon
leaf
pod
CHLOROPHYLL
1·6
0·8
0
mg·g FRESH WT.⁻¹
leaf
pod
cotyledon
6
12
18
24
30
AGE (DAYS)

FIGURE 3

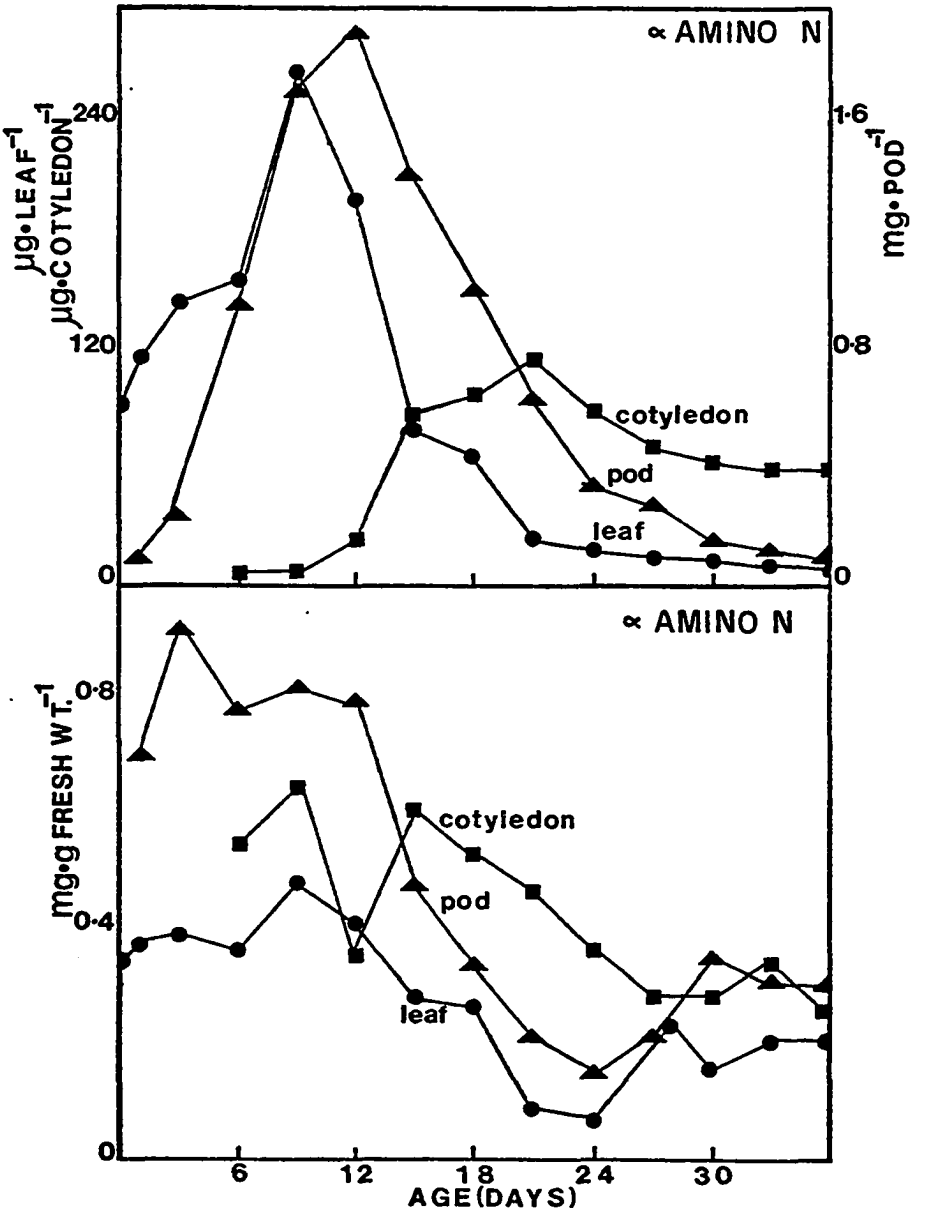
∝ AMINO N
µg·LEAF⁻¹
µg·COTYLEDON⁻¹
240
120
0
1·6
0·8
0
mg·POD⁻¹
cotyledon
pod
leaf
∝ AMINO N
mg·g FRESH WT.⁻¹
0·8
0·4
0
cotyledon
pod
leaf
6
12
18
24
30
AGE(DAYS)

FIGURE 4

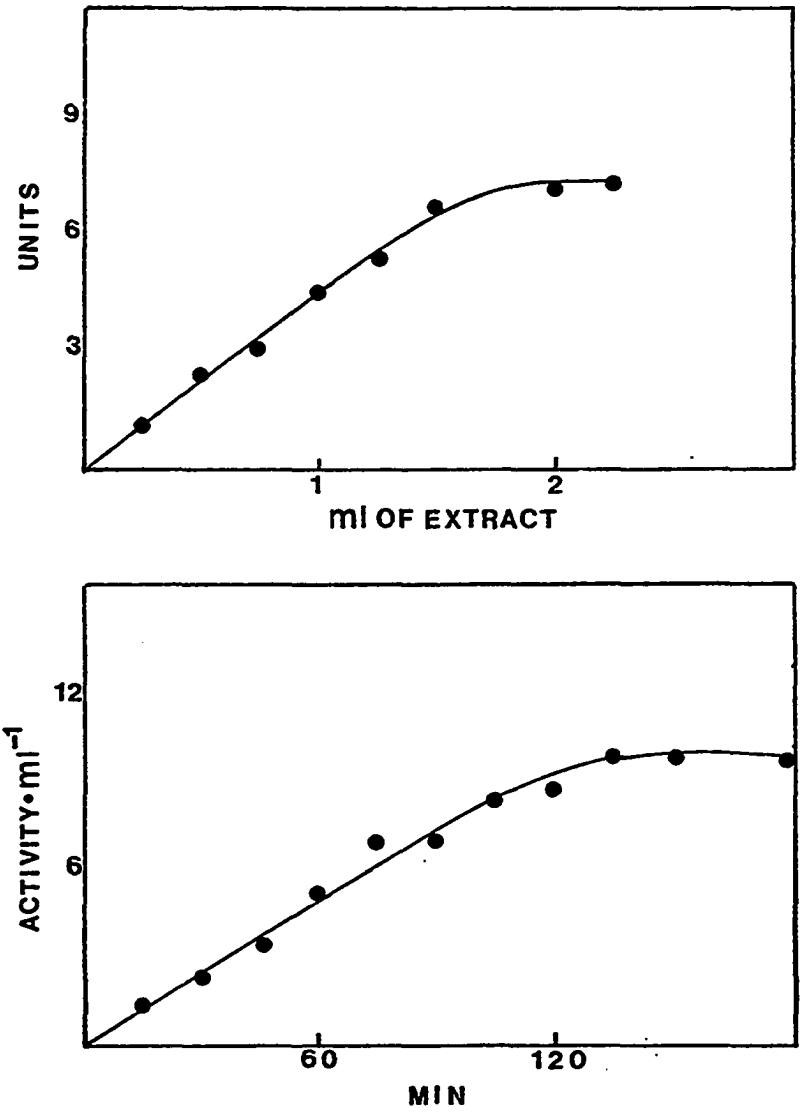

FIGURE 5

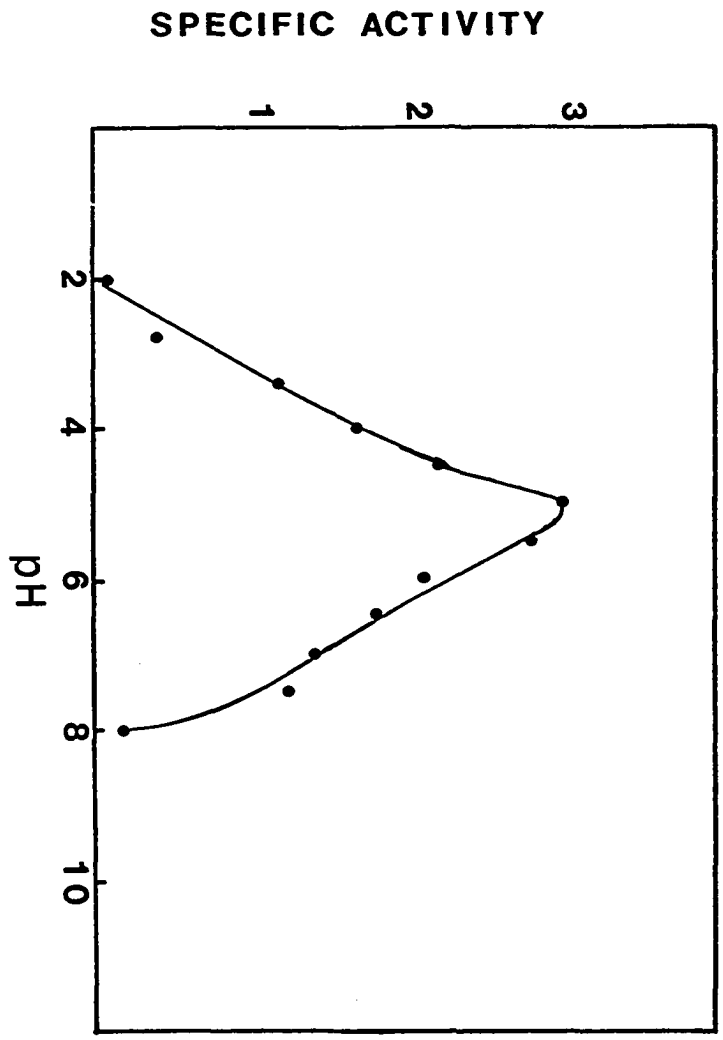

FIGURE 6

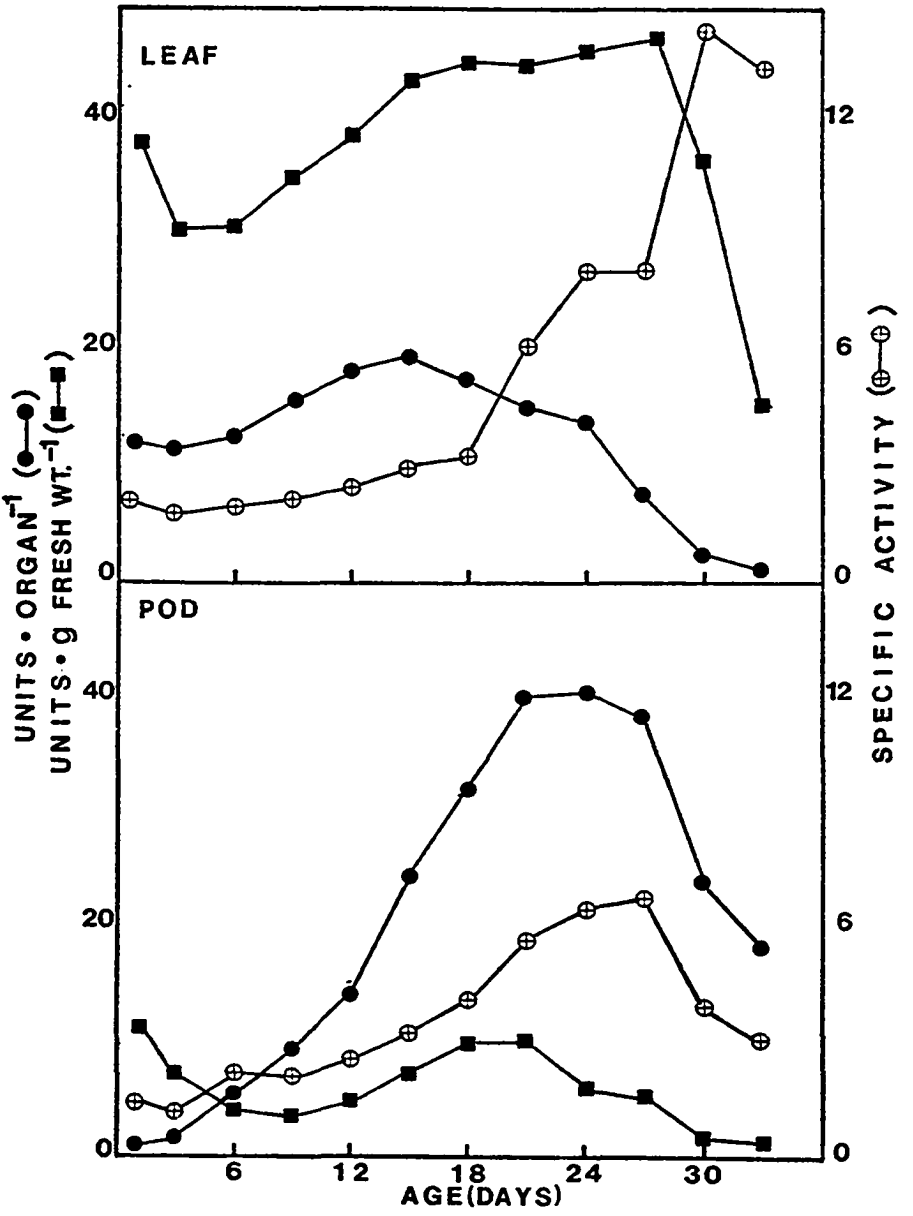
LEAF
POD
40
20
0
12
6
0
UNITS • ORGAN^{-1} (●—●)
UNITS • g FRESH WT.$^{-1}$ (■—■)
SPECIFIC ACTIVITY (⊕—⊕)
6
12
18
24
30
AGE(DAYS)

FIGURE 7

CHAPTER IV

PAPER 3

ENZYMOLOGY OF GLUTAMINE METABOLISM RELATED TO SENESCENCE AND SEED DEVELOPMENT IN THE PEA (*PISUM SATIVUM* L.)

ABSTRACT

The metabolism of glutamine in the leaf and subtended fruit of the ageing 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.2.6.1.2] 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. These enzymes were studied to determine their roles in amide metabolism for nitrogen economy in the fruiting pea plant. Maximal glutamine synthetase activity and free amino acid accumulation occurred together in the young leaf. Glutamine synthetase (in vitro) in leaf extracts exceeded the requirement (in vivo) for reduced nitrogen in the organ. Glutamine synthetase activity, although declining in the senescing leaf, was sufficient (in vitro) to produce glutamine from all the N released during protein hydrolysis (in vivo). Maximal glutamine synthetase activity in the pod was recorded 6 days after the peak accumulation of free amino acids in this organ. Glutamine synthetase in both the leaf and pod was associated with the chloroplast but considerable soluble activity was also detected.

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 glutamate synthetase activity was recorded simultaneously with maximal pod glutamine synthetase.

It is suggested that the activity of glutamine synthetase in the supply organs (leaf, pod) could furnish the translocated amide necessary for the nitrogen 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.

INTRODUCTION

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, 28). 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 carbon and nitrogen imported by the seed via the phloem (21, 23, 24, 25, 39, 40, 41). Most of the nitrogen translocated to the ripening fruit is in the amides, glutamine and asparagine (2, 7, 21, 24, 36, 37, 39, 40) and they are the main nitrogen donors for in situ synthesis of protein amino acids in the seed (21, 23, 24, 25, 40). The importance of amides in the nitrogen 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 (29, 45, 53).

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 nitrogen imported by the developing cotyledons (7, 21, 23, 36, 37, 38). Pate et al. (40) 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 carbon fixation. The pod is almost totally committed to the nitrogen nutrition of its developing ovules (18, 24, 30, 41, 43).

In a previous communication, we demonstrated the operation of a leaf and pod proteolytic activity capable of releasing amino acids from the protein of ageing supply organs (51). In this paper we report the presence of glutamine synthetase (EC.6.3.1.2) and glutamate synthetase (EC.2.6.1.53) activity in ageing supply and developing recipient organs of

the pea. The respective catalytic potentials of these enzymes to incorporate nitrogen into translocated glutamine and subsequently utilize this nitrogen for assimilatory growth in the cotyledons are crucial to the nitrogen economy of the plant.

MATERIALS AND METHODS

Plants (Pisum sativum L. cv. Burpeeana) were grown under controlled environmental conditions and the age (days post anthesis) of individual organs was followed as before (51). 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, chlorophyll and soluble alpha amino nitrogen were made as described previously (51). 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 (31). All steps were carried out at 5C or less. The extraction medium was 50 mM Pipes [piperazine-N,N'bis (2-ethane-sulfonic acid) monosodium salt, monohydrate] buffer (pH 6.8) containing 0.33 M sorbitol, 2mM $NaNO_3$, 10 mM 2-mercaptoethanol, 1 mM $MnCl_2$, 2 mM EDTA, 2 mM sodium arsenate, 4 mM sodium ascorbate and 0.1 % BSA. One gram of tissue was added to 4 ml extraction medium and then completely mascerated with a mechanical razor blade chopper (4). The resulting mixture was filtered through miracloth (Calbiochem) into a conical test tube then centrifuged at 600 g for 10 minutes. 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 (15 X 20 cm) as described previously (51). 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 BSA. The protein rich fractions, which eluted with the void volume, were pooled, analyzed for soluble amino acids (see 51) and assayed for glutamine synthetase activity.

Glutamine synthetase, unless stated otherwise, was measured as glutamyl transfer activity by a modification of the methods of O'Neal and Joy (33) and Varner and Webster (54). 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 3.0 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 5000 g for 10 min. The clarified supernatant was analyzed for the presence of gamma glutamyl hydroxamate (GHA) by spectrophotometric determination (540 nm) of its ferric chelate (9, 47). 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 (13, 47). Synthesis of GHA during the course of the reaction (as determined from the standard curve) was taken to indicate glutamine synthetase activity. One unit of activity is defined as the amount of enzyme which catalyzed the formation of 1 µmol gamma glutamyl hydroxamate $\cdot$ min^{-1} under the standard conditions described above. Specific activity is units $\cdot$ mg^{-1} protein in the enzyme extract.

Protein was determined by the method of Lowry et al. (26). Chlorophyll was measured in 80% (v/v) acetone by the method of Arnon (1).

Extracts from 6, 12, 18 and 24 day leaves and 12, 18 and 24 day pods were also assayed for glutamine synthetase activity by the biosynthetic method described by O'Neal and Joy (32) 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 (57) using a reaction mix similar to that of the biosynthetic assay (32). Enzyme extracts from the G-50 column (materials and methods) were incubated in 0.1 MTricine-KOH, 20 mM $MgSO_4$, 1mM 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 minutes. Similar incubations were made with reaction mixtures minus glutamate, NH_4Cl and ATP. The course of the synthesis reaction was terminated by addition of 4 ml ice cold acetone and the precipitated protein was removed by centrifugation. An aliquot of the resulting supernatant was applied to Whatman 3 MM 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).

The pH optimum for glutamyl transfer activity by glutamine synthetase 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.

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 buffer (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 - 100 µl of 100,000 g supernatant was assayed. One unit of glutamate synthetase activity is the amount of enzyme which catalyzes the oxidation of 1 nmol NADH · min^{-1} at pH 7.5, 30 C. Specific activity is units · mg $protein^{-1}$ in the enzyme extract. Protein was determined on trichloroacetic acid (TCA) precipitates by the method of Lowry et al. (26). 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 glutamine synthetase or glutamate synthetase) containing different levels of enzymatic activity and from separate developmental stages, were

brought to equal protein concentrations [protein determined by the method of Warburg et al. (56)] then mixed in proportions of 0, 25, 50, 75 and 100 per cent of each extract. The respective mixtures were assayed for enzymatic activity as described above.

RESULTS

Growth and Development. Growth patterns (fresh weight) 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 5 to 6 mature 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, the leaf, then pod matured, and both lost weight during the period of intensive cotyledonary growth (day 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 Ageing. Figure 2 represents the developmental changes in chlorophyll, protein and soluble α amino nitrogen (amino acids) in the leaf, pod and cotyledon. Early increases in leaf (day 0-9) and pod (day 3-12) chlorophyll were recorded, but the level decreased after full expansion of each organ. Protein content in the leaf and pod increased until day 12 and 15 respectively, then decreased while protein accumulated in the developing cotyledons (day 12-27). During expansion, free amino acids accumulated in the leaf (day 0-9) and pod (day 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.

Enzymatic activity. The optimum conditions for measuring glutamine synthetase transfer activity in leaf and pod extracts were determined. Transfer activity was linear with time (up to 8 min) and amount of enzyme extract assayed (up to 200 µl) (Fig. 3). Optimal transfer activity occurred at pH 6.8 (Fig. 4). 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. 5). No synthesis of glutamine was detected when NH_4Cl, ATP or L-glutamate were absent from the assay mixture.

The biosynthetic activity of glutamine synthetase (32) in crude pea leaf or pod extracts was also measured. In this assay, the formation of gamma glutamyl hydroxamate from L-glutamate and hydroxylamine was linear with time and amount of enzyme extract added to the reaction mixture at pH 7.8, 30 C (data not shown). Determinations of glutamine synthetase activity by the transfer method were consistently more sensitive (9-10 fold) than those by the biosynthetic method. O'Neal and Joy (33) reported a similar 7 to 8 fold differential sensitivity with purified glutamine synthetase from pea leaves. In addition, the cellular distribution of enzyme activity could be measured by either method with similar (ratio) results (31). Other workers have compared the relative activities of the two assays and found their ratios remained constant either during purification steps (8, 9, 54), as a function of time by crude and purified preparations (35) or as a function of growth on various nitrogen sources (44). Attempts to separate the two activities in plant extracts have not been successful (8).

The optimum conditions for measuring glutamate synthetase 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 glutamine synthetase activity are shown in Figure 6. Activity was detected by the transfer assay because of its greater sensitivity and ease of measurements; it is also less subject to interference by contaminating enzymes in crude cell extracts (49). Developmental activity is reported as a function of fresh weight and specific activity to facilitate comparison to other studies. However, as suggested previously (51), it is more meaningful to consider developmental data as a function of a constant parameter that does not itself fluctuate with time. Therefore, data in this paper is also quoted, and mainly discussed, on a per organ basis.

Leaf glutamine synthetase activity increased with leaf expansion (to day 9) and reached a maximum at day 12 (46.5 units · $organ^{-1}$)(Fig. 6). 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 chlorophyll level (Fig. 2) and glutamine synthetase activity (Fig. 6) were similar and the per cent of activity associated with the chlorophyll enriched fraction of crude leaf extracts was relatively constant (24-31%) until final senescence (day 30) of the organ (Table 1). This degree of association of p leaf glutamine synthetase activity with the chloroplast is in agreement with that reported by O'Neal and Joy (31). Activity increased simultaneously

with the level of free amino acids in the young leaf and then both decreased at near equal rates during leaf senescence (Fig. 2,6). Specific activity and activity$\cdot g^{-1}$ fresh weight were relatively constant throughout development with a slight peak at day 12 followed by a constant slow decrease until day 33. (Fig. 6).

Pod glutamine synthetase activity increased rapidly during early growth, then reached a maximum (48.1 units $\cdot$ organ^{-1}) 12 days after elongation ceased. (Fig. 6). Thereafter, activity was rapidly lost until day 30. Average activity throughout the 33 day maturation period was 26.1 units$\cdot$ day^{-1}. As with the leaf, pod activity was associated with the chloroplast enriched fractions of crude extracts (Table 1). Maximum activity was recorded 6 days after chlorophyll had begun to steadily disappear from the organ (Fig. 2,6). A rapid loss of the soluble amino acids from the pod also began 6 days prior to maximum activity (Fig. 2,6). There was little fluctuation in specific activity or activity $\cdot$ g^{-1} fresh weight during development with a slight decrease during senescence of the pod (Fig. 6).

Numerous regulatory mechanisms are known to influence glutamine synthetase activity in plant extracts (12, 17, 32-34, 54). Thus, it was possible extracts from separate developmental stages of the leaf or pod contained components which influenced individual <u>in vitro</u> assays for transfer activity. Evidence against this possibility was obtained by mixing extracts from separate developmental stages which exhibited different levels of glutamine synthetase activity. Assays on the these mixtures yielded activities which were the average of the individual extracts (Table 2), thus indicating no apparent regulator of glutamine synthetase activity was present in either extract.

Glutamine synthetase activity was also detectable in extracts from developing cotyledons (Table 3) but at a much lower level than in leaf or pod extracts (Fig. 6). Cotyledonary glutamine synthetase 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 (20). O'Neal and Joy (32) 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 glutamine synthetase for purification and characterization of the plant enzyme (8, 9, 17, 54, 57). These workers began with large quantities of seeds (18 kg, approximately 10^4 cotyledons) and recovered equally low or lower levels of specific activity in crude extracts as reported here.

Glutamate synthetase activity was detected in extracts of developing pea pods (Fig. 7). Activity increased rapidly as the pod elongated, then 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 developmental changes in protein and free amino acid content in the pod (Fig. 2, 7). Maximum pod glutamate synthetase activity was recorded on the same day as that of leaf glutamine synthetase, but 6 days prior to the maximum of pod glutamine synthetase (Fig. 6, 7). The specific activity of glutamate synthetase 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. 7).

Cotyledonary glutamate synthetase 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. 7). Average activity was 43 units · cotyledon^{-1} · day through development and maturation. Free amino acids and then reserve proteins accumulated as glutamate synthetase activity increased in the developing cotyledon (Fig. 2, 7). Maximum glutamate synthetase activity was recorded 3 days after maximum glutamine synthetase activity in the pod and 9 days after that in the leaf (Fig. 6, 7). However, the initial increase in cotyledonary glutamate synthetase activity paralleled the initial increase of glutamine synthetase activity in both supply organs (Fig. 6, 7). The specific activity of glutamate synthetase increased tremendously from day 6 to 12 in the ripening ovule but decreased rapidly as reserve protein accumulated (Fig. 2, 7). Activity on a fresh weight basis declined rapidly with the loss of specific activity (Fig. 7).

Mixing experiments were also conducted with extracts of different age organs exhibiting contrasting levels of glutamate synthetase activity (Table 4). 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 nitrogen gained and lost in each organ (Table 5). Each ripe fruit contained 5 to 6 mature seeds (average of 11 cotyledons · fruit^{-1}) representing a total of 143 mg cotyledonary reserve protein (22.9 mg N) per fruit. Each pod lost 22 mg protein (3.5 mg N) during senescence. If,

as reported, all of the protein lost from the pod was exported to the developing ovules in the fruit (see 18, 24, 36, 37, 40, 41, 43) it would constitute 15.3% of the nitrogen in the mature cotyledons. Recently, Pate et al. (41) demonstrated that 16% of the lupine seed's nitrogen had been mobilized from the senescing pod. Ten mg protein was lost from the leaf. It is evident that there was insufficient protein nitrogen assimilated in, then mobilized from the leaf and pod of the lowest fruiting node, to account for all the protein nitrogen deposited in the 5 or 6 seeds of the fruit.

Enzymatic Potential During Ontogeny. Calculations based on in vitro measurements of enzymatic activity (Fig. 6, 7) were made to assess the in vivo catalytic potential for glutamine metabolism during the life of each organ (Table 5). These estimates may be compared to actual (in vivo) gain and loss of protein nitrogen, in the ageing organ (Fig. 2 and above). Leaf glutamine synthetase activity (in vitro) was sufficient to have previously assimilated all the nitrogen into glutamine which was subsequently metabolized, then incorporated into the accumulated protein of the organ (day 0-12). There was also ample potential to assimilate into glutamine all the amino nitrogen which was released from leaf protein during senescence (day 13-33). Moreover, leaf glutamine synthetase activity (in vitro) during the life of the subtending organ was adequate to have assimilated into glutamine (for export) all of the nitrogen imported by the fruit (of the same node) and present in the protein and amino acids of the reproductive structure (pod plus 6 seeds) at senescence (day 33).

The activity of pod NAD(P)H dependent glutamate synthetase (in vitro) was sufficient (28 fold) to account for the metabolism (via imported glutamine to glutamate then transamination reactions) of all the nitrogen incorporated into pod protein (Table 5). It is recognized that a ferredoxin

dependent system of glutamate synthesis (such as occurs in the leaf, see 27) might have operated in the pod. But there was relatively little chlorophyll 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 (14, 16, 37, 42). Because of the efficiency of the NAD(P)H dependent enzyme in the pod, assays for activity requiring ferredoxin were not conducted in this developmental study.

In the ripening ovules, there was sufficient (10 fold) *in vitro* catalytic potential for all of the nitrogen present in reserve proteins (*in vivo*) to have been previously metabolized through the activity of cotyledonary glutamate synthetase (Table 5).

DISCUSSION AND CONCLUSIONS

The changes in leaf glutamine synthetase activity during ontogeny appear to coincide with changes reported for respiration, photosynthesis and other metabolic activities in ageing pea leaves (7, 11, 36, 37). It has been shown that photosynthetic capacity reached a maximum just after full expansion of the pea leaf (10,11) and, in supply leaves of the reproductive node, could be maintained at near maximum for several days (37). Others have reported an accumulation of free amino acids in the young, expanding field pea leaf and found the reduction of nitrate in the leaf was most active during this period of growth (7, 36, 37). Wallace and Pate (55) speculated that the amides may be the immediate organic products of nitrate reduction and later, Pate et al. (40) found that the biosynthesis of amides in leaves was closely related to the reduction of nitrate. Glutamine has been shown to be the major acceptor of photosynthetically reduced nitrogen in the leaf (21,22) and the capacity to utilize this assimilated nitrogen for subsequent amino acid biosynthesis in the organ has been reported (21, 25, 40). Further evidence in support of a relationship between nitrogen reducing enzymes and glutamine synthetase in the leaf has been discussed by Miflin and Lea (27). The currently recorded accumulation of free amino acids and increasing glutamine synthetase activity in the young pea supply leaf (Fig. 2, 6) is in harmony with the observations cited above and supports the contemporary proposal that glutamine synthetase is involved in the physiological mechanism of nitrogen assimilation in the leaf (see Miflin and Lea (27) for a review). This is probably a major role of glutamine synthetase in the pea leaf.

We found that *in vitro* activity of leaf glutamine synthetase exceeded the *in vivo* requirements for reduced nitrogen in leaf protein synthesis (Table 5). 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 ageing organ (40, 51). Speculation in support of this scheme was provided by Lea and Fowden (20) 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, glutamine synthetase could then readily produce glutamine for direct export or subsequent amide metabolism. Because it is known that the amides represent the bulk of translocated nitrogen and that their concentration in the phloem increases with leaf age (7, 25, 38, 39, 40, 51), it might be expected that leaf glutamine synthetase activity would also increase during senescence. This was not the case (Fig. 6). However, *in vitro* estimates of endogenous glutamine synthetase activity indicate there was ample catalytic potential present in the ageing leaf (day 13-33) to metabolize all the leaf protein hydrolysates to glutamine (Table 5). It is suggested that this is another role of glutamine synthetase in the pea leaf.

Glutamine synthetase activity in the pod was probably not involved in the assimilation of photosynthetically reduced nitrogen. Other workers have reported that little or no nitrate was presented to the ageing lupine pod (39, 41) because it was reduced in the subtending leaf (40). Schlesier and Muntz (48) found low levels of bean pod nitrate reductase activity that decreased as the fruit aged. In contrast, maximum glutamine synthetase activity of the pea pod (Fig. 6) was recorded during senescence.

The most likely role of pod glutamine synthetase is the assimilation of nitrogen released in the fruit through deamidation of imported amides or oxidation 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 (51). This increase occured at the same time as the increase in pod glutamine synthetase activity reported here (Fig. 7). In vitro estimates of pod glutamine synthetase activity indicate the presence of ample in vivo potential to metabolize the nitrogen released during proteolysis (Table 5).

A mechanism for the transfer of imported glutamine amide nitrogen to alpha amino nitrogen is provided by the activity of glutamate synthetase. Activity in the pod increased to its maximum during the time of rapid growth and assimilation in the organ but prior to such activities in the ovule. This period of pea ontogeny was also marked by the maximum activity of leaf glutamine synthetase. 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 (see 30)) the amide synthesized in excess in the leaf could be translocated to the pod and metabolized by glutamate synthetase. This amino nitrogen could then be utilized in pod protein synthesis which also shows a marked increase during this period (Fig. 2). Additionally, it is possible glutamine and glutamate synthetase in the pod operate in an associated manner similar to that proposed for the leaf of the plant (27). However, 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 glutamate synthetase activity (Fig. 6) corresponds to that reported by others for PEP carboxylase activity in the young pea pod (16). This enzyme is involved in the

reassimilation of CO_2 respired by the developing pea seed (14, 16), especially in the early life of the fruit (10, 11, 14), Kipps and Boulter (18) 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 nitrogen donated from glutamate through the activities of aspartate aminotransferase (15). We suggest that the glutamate required in this system could be supplied through the activities of pod glutamate synthetase metabolizing the glutamine imported from the leaf during the early life of the pod.

Glutamate synthetase activity in the developing cotyledons increased during growth and nitrogen accumulation in the organs (Fig. 2). This activity corresponds to the time of maximum pod glutamine synthetase activity and to the disappearance of protein and free amino acids from the leaf, then pod (Fig. 2, 6). It has been suggested that these amino acids are translocated to the ripening ovule as amides (7, 37, 38, 40, 51) and that the function of glutamate synthetase 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 5) and the developmental evidence provided in this paper supports the earlier conclusion.

An extremely low level of glutamine synthetase activity was present in the developing pea cotyledons (Table 3). Thus it would appear that this limited assimilatory capacity would be insufficient to utilize free ammonia such as that which might be released by amidohydrolase activity on imported glutamine or asparagine. The failure to detect glutaminase or asparaginase (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 nitrogen metabolism and donates amide nitrogen to numerous substrates (20, 50). 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 (19, 20, 46, 52). The operation of this glutamine requiring enzyme would add additional 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. Even more perplexing is the almost total lack of information concerning the enzymes of asparagine utilization (2, 19, 20, 27). Studies into these mechanisms are proceding in our laboratory.

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TABLE 1. Glutamine synthetase activity in chlorophyll enriched fractions of leaf and pod extracts.

Leaf				Pod			
	% of total in 600 g pellet				% of total in 600 g pellet		
Age days	Chl	Protein	GS	Age days	Chl	Protein	GS
1	74	31	24	1	79	42	41
3	70	30	24	3	77	42	37
6	74	36	21	6	77	41	38
9	67	31	24	9	65	39	39
12	66	38	23	12	66	42	39
15	68	38	25	15	66	45	40
18	70	39	24	18	71	49	42
21	78	42	26	21	79	45	44
24	80	42	27	24	73	45	42
27	70	34	31	27	75	37	42
30	78	36	45	30	75	39	54
33	77	35	45	33	71	33	59
Avg	73	36	28	Avg	73	43	42

Leaf and pod glutamine synthetase (GS) were extracted and assayed under standard conditions as described in materials and methods. Results are the mean of triplicate assays on three independently prepared extracts and similar rates of catalysis were measured from each assay. Total values are show in Fig. 2 and 6.

Table 2. Glutamine synthetase mixing experiments.

Leaf			Pod		
% of extract in assay mix		Specific	% of extract in assay mix		Specific
Day 12	Day 30	Activity	Day 18	Day 30	Activity
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

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 then assayed under standard conditions. Values given are the mean of triplicate assays from two independently prepared extracts. Specific activity = μmol gamma glutamyl hydroxamate formed $\cdot$ min^{-1} . mg protein.

TABLE 3. Glutamine synthetase activity in developing pea cotyledons.

Age (Days)	6	12	18	24	30	Mature
Units · Cotyledon^{-1}	ND	0.01	0.106	0.138	0.248	0.292
Specific Activity	ND	0.011	0.028	0.011	0.018	0.023

Extracts were prepared as discussed in the text. Transfer assays were conducted under standard conditions (Materials and Methods). 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 samples. Mature cotyledons were taken from dry, dehisced fruits, 36 to 40 days old (post anthesis). Units = μmol gamma glutamyl hydroxamate formed · min^{-1}. Specific activity = units ·mg^{-1} protein in assay. ND = not detected.

Table 4. Glutamate synthetase mixing experiments.

Pod			Cotyledon		
% of extract in assay mix		Specific	% of extract in assay mix		Specific
Day 9	Day 27	Activity	Day 15	Day 27	Activity
100	0	31.5	100	0	78.8
75	25	33.0	75	25	63.2
50	50	35.9	50	50	44.1
25	75	38.5	25	75	24.4
0	100	41.0	0	100	6.1

Enzyme extracts of equal protein concentration were prepared as described in the text. The extracts were combined in the concentration indicated and then assayed under standard conditions. Values given are the mean of triplicate assays from two independently prepared extracts. Specific Activity = nmol NADH exidized · min^{-1} · mg protein.

Table 5. Nitrogen metabolism in the ageing leaf, pod and cotyledon.

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

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 (see Fig. 6, 7) to determine loss of protein N from each organ (see 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 spectrometrically, where 1 nmol NADH oxidized = 1 nmol glutamine consumed = 2 nmol glutamate produced. GOGAT is expressed as mg amide N potentially metabolized · organ^{-1} over the developmental period (day 1-12, 13-33, total) indicated. See Fig. 6, 7 for developmental profile of enzyme activities. To account for $\simeq$ in growth chamber vs. assay temperature, the activity of each enzyme was reduced by a $Q_{10} = 2$. Nitrogen in free amino acids was not included in the calculations to determine the N budget of each organ because it represented $\geq$1 % of the protein N. [1]Total for all cotyledons in the fruit.

LEGENDS FOR FIGURES

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 non-nodulated pea plant. Age is days post anthesis.

Fig. 2. Developmental changes in chloroyhyll, protein and soluble alpha amino nitrogen (∝ amino N) levels in the leaf, pod and cotyledon of the pea. Age is days post anthesis.

Fig. 3. The linear relationship of glutamine synthetase transfer activity to time and amount of enzyme extract in the assay mixture. Data shown is for extracts of 15 day old leaves prepared and assayed by standard methods (materials and methods). Similarly shaped curves were also obtained for each developmental stage of the leaf, pod and cotyledon. Units = μmol GHA produced $\cdot$ min^{-1}. Activity = μmol GHA produced.

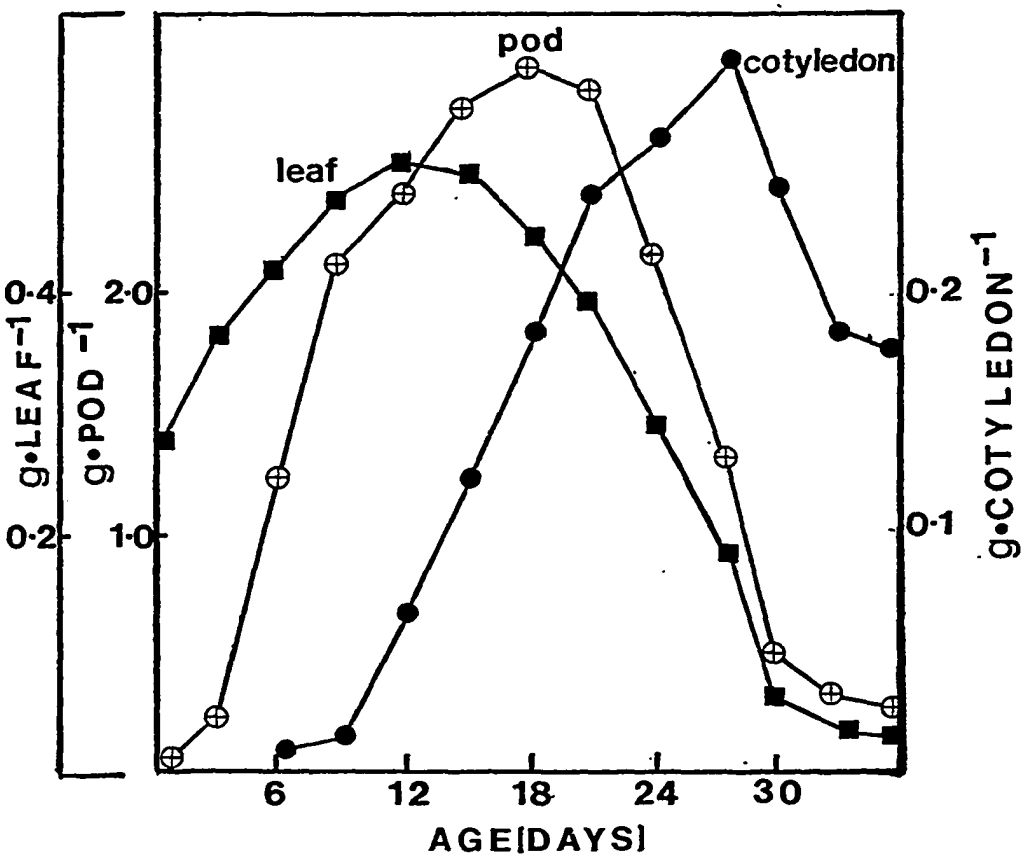
Fig. 4. Effect of pH on transfer activity of glutamine synthetase. Tissue from 15 day leaves was extracted and assayed as a function of pH as described in materials and methods. Activity was linear with time and amount of extract in the reaction mixture. Similar symmetrical pH curves were obtained from assays of other developmental stages of the leaf, pod and cotyledon. Specific activity = μmol GHA formed $\cdot$ min^{-1}.mg protein in extract.

Fig. 5. Paper chromatography of the reaction products from assays for glutamine synthetase activity in crude extracts. Extracts of 12 day pea leaves were prepared and assayed as described in the text. Similar results were obtained with extracts of 18 day old pods. The complete assay mixture was enzyme extract in 0.1M Tricine - KOH buffer containing 20 mm $MgSO_4$, 1 mM EDTA, 10 mM ATP, 8mM 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.

Fig. 6. Levels of glutamine synthetase 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 post anthesis. Organs were harvested from the first fruiting node only. Leaf = leaflet plus stipule; pod = carpel wall. Units = μmol GHA formed · min^{-1} at pH 6.8, 30C. Specific activity = units·mg $protein^{-1}$ in reaction mix.

Fig. 7. Levels of glutamate synthetase activity in extracts from developing pea pods and cotyledons. Activity was measured under optimum conditions as described in the text. Age is days post anthesis. Units = nmol NADH oxidized · min^{-1} at pH 7.5, 30C. Specific activity = units · mg $protein^{-1}$ in reaction mix.


pod
cotyledon
leaf
0·4
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0·2
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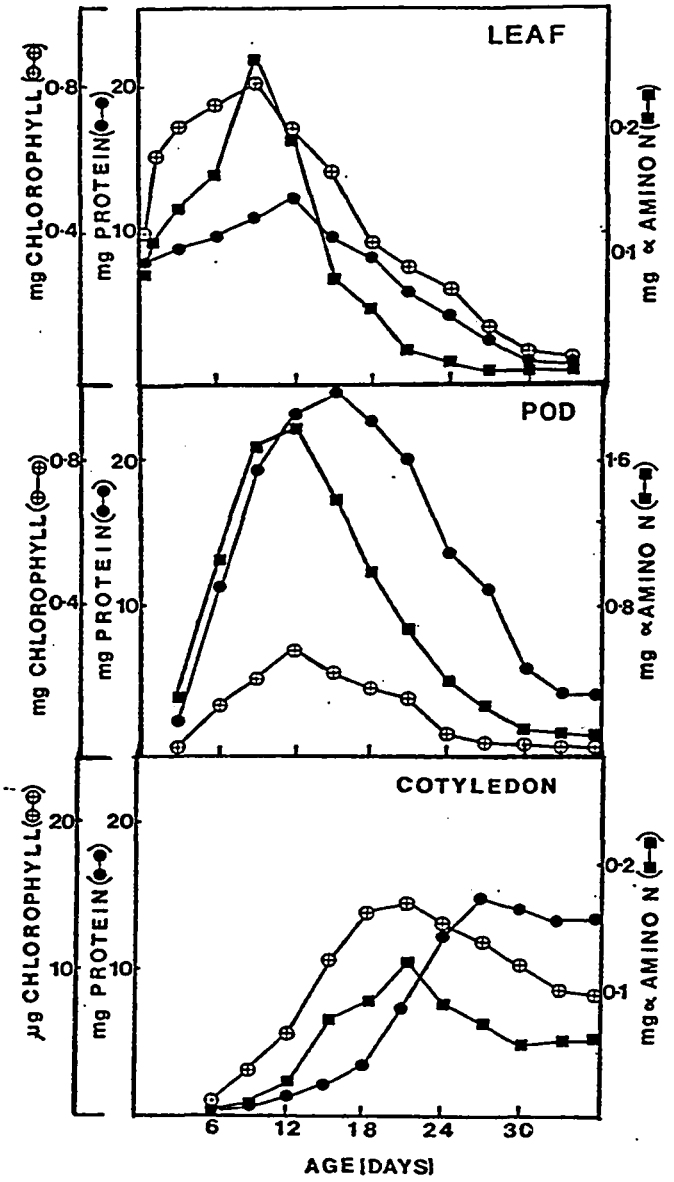
g•LEAF⁻¹
g•POD⁻¹
g•COTYLEDON⁻¹
6
12
18
24
30
AGE(DAYS)

FIGURE 1


LEAF
POD
COTYLEDON
mg CHLOROPHYLL
mg PROTEIN
mg ∝ AMINO N
µg CHLOROPHYLL
mg ∝ AMINO N
0·8
0·4
20
10
0·2
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1·6
0·8
6
12
18
24
30
AGE (DAYS)

FIGURE 2

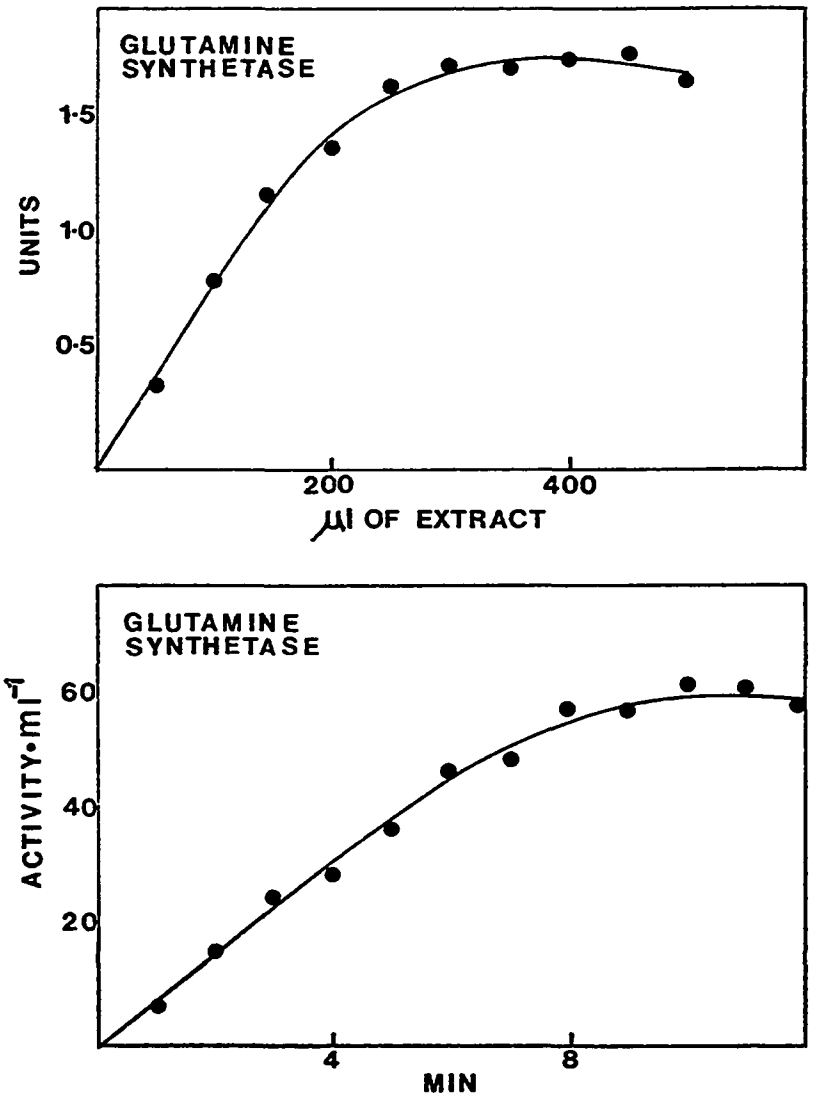
GLUTAMINE
SYNTHETASE
UNITS
1·5
1·0
0·5
200
400
µl OF EXTRACT
GLUTAMINE
SYNTHETASE
ACTIVITY·ml⁻¹
60
40
20
4
8
MIN

FIGURE 3

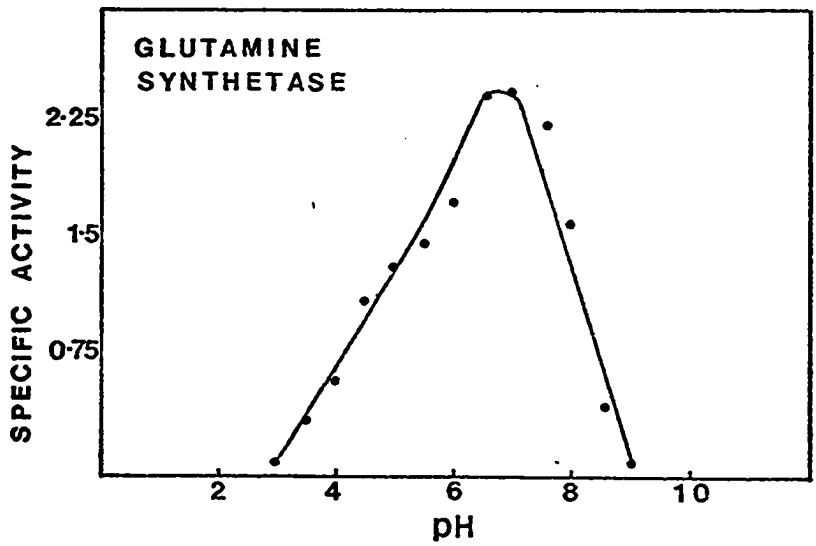
GLUTAMINE
SYNTHETASE
SPECIFIC ACTIVITY
2·25
1·5
0·75
2
4
6
8
10
pH

FIGURE 4

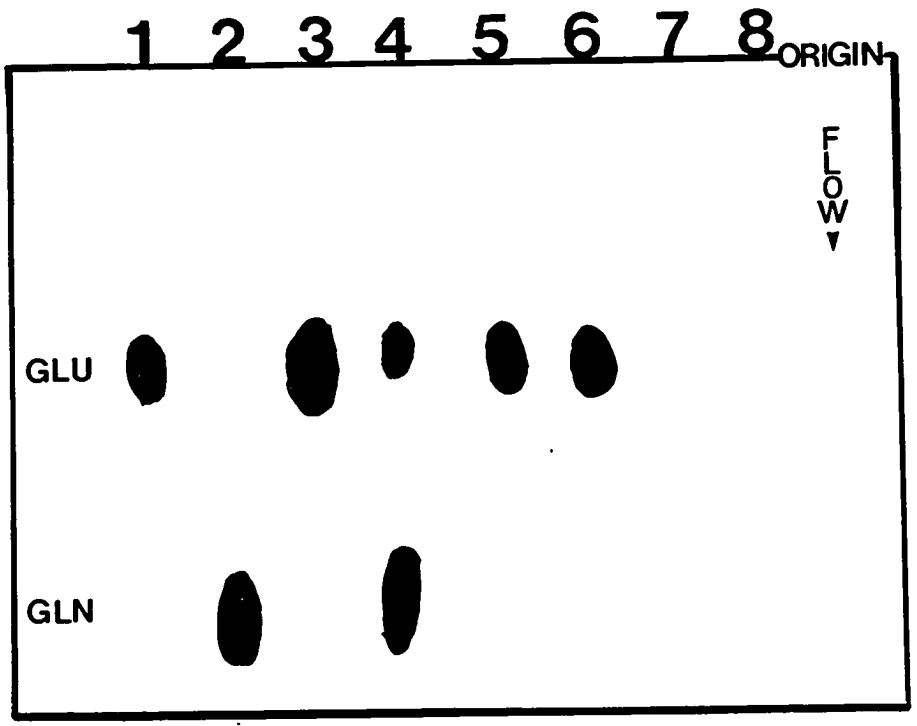

FIGURE 5

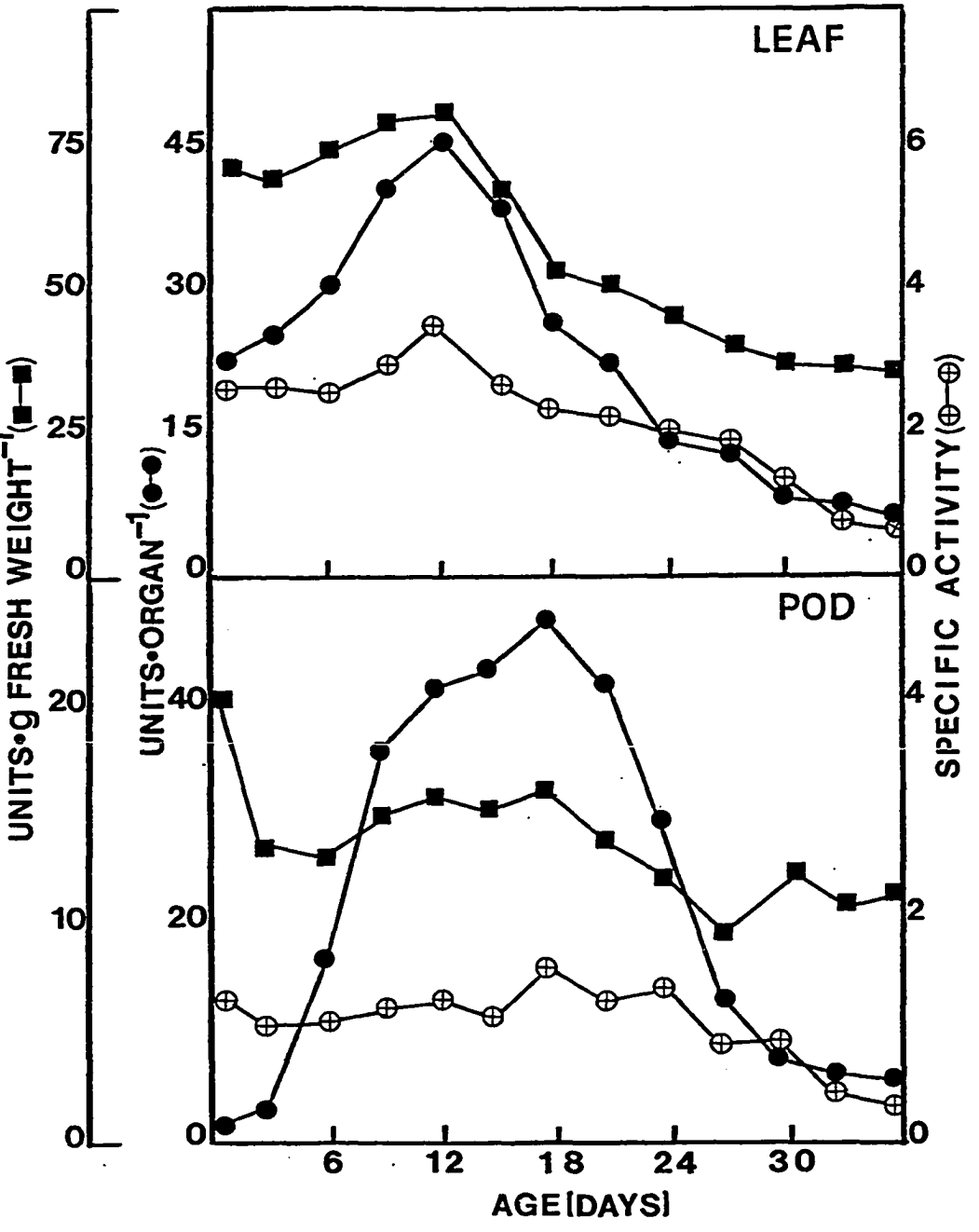
LEAF
POD
UNITS•g FRESH WEIGHT^{-1}(■—■)
UNITS•ORGAN^{-1}(●—●)
SPECIFIC ACTIVITY(⊕—⊕)
AGE (DAYS)
0
25
50
75
15
30
45
2
4
6
10
20
40

FIGURE 6