MICROCOPY RESOLUTION TEST CHART
NATIONAL BUREAU OF STANDARDS
STANDARD REFERENCE MATERIAL 1010a
(ANSI and ISO TEST CHART No. 2)

National Library
of Canada

Bibliothèque nationale
du Canada

Canadian Theses Service

Services des thèses canadiennes

Ottawa, Canada
K1A 0N4

CANADIAN THESES

THÈSES CANADIENNES

NOTICE

The quality of this microfiche is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us an inferior photocopy.

Previously copyrighted materials (journal articles, published tests, etc.) are not filmed.

Reproduction in full or in part of this film is governed by the Canadian Copyright Act, R.S.C. 1970, c. C-30.

AVIS

La qualité de cette microfiche dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de qualité inférieure.

Les documents qui font déjà l'objet d'un droit d'auteur (articles de revue, examens publiés, etc.) ne sont pas microfilmés.

La reproduction, même partielle, de ce microfilm est soumise à la Loi canadienne sur le droit d'auteur, SRC 1970, c. C-30.

**THIS DISSERTATION
HAS BEEN MICROFILMED
EXACTLY AS RECEIVED**

**LA THÈSE A ÉTÉ
MICROFILMÉE TELLE QUE
NOUS L'AVONS REÇUE**

DIURNAL VARIATION OF ASPARAGINASE IN DEVELOPING PEA LEAVES

- by

KONRAD ANDREW SIECIECHOWICZ

A thesis submitted to
the Faculty of Graduate Studies and Research
in partial fulfilment of
the requirements for the degree of
Doctor of Philosophy

Department of Biology

Carleton University

Ottawa, Ontario

July 3 1986

© copyright

1986, K.A. Sieciechowicz

Permission has been granted to the National Library of Canada to microfilm this thesis and to lend or sell copies of the film.

The author (copyright owner) has reserved other publication rights, and neither the thesis nor extensive extracts from it may be printed or otherwise reproduced without his/her written permission.

L'autorisation a été accordée à la Bibliothèque nationale du Canada de microfilmer cette thèse et de prêter ou de vendre des exemplaires du film.

L'auteur (titulaire du droit d'auteur) se réserve les autres droits de publication; ni la thèse ni de longs extraits de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation écrite.

ISBN 0-315-33383-9

The undersigned hereby recommend to
The Faculty of Graduate Studies and Research
acceptance of the thesis,
"Diurnal variation of asparaginase in developing pea leaves"
submitted by
Konrad Andrew Sieciechowicz
in partial fulfilment of the requirements
for the degree of Doctor of Philosophy

.....
Chairman, Department of
Biology

.....
Thesis Supervisor

.....
External Examiner

Carleton University

July 31 1986

Abstract

This study examined the hydrolysis of asparagine (a major nitrogen transport compound in many plants), by asparaginase, in developing pea leaves. Asparaginase activity varied diurnally in leaves that were rapidly accumulating nitrogen in soil or hydroponically grown plants; maximum activity was observed during the light period. The greatest increase in activity (and decrease during the dark period) was found in half expanded leaves; no diurnal variation was observed in very young or mature pea leaves. When assayed at the end of the dark period, asparaginase activity decreased with leaf age. Asparaginase activity, in successive young or half expanded leaves, increased with plant age. An increase in asparaginase activity was noted in detached half expanded leaves obtained from soil-grown plants when fed for 2.5 to 4 hours in the light. However, no increase was observed in detached, half expanded hydroponically-grown leaves under the same conditions. The light-induced increase in asparaginase activity was sensitive to MSO, an inhibitor of glutamine synthetase activity. Increased asparaginase activity in half expanded leaves was dependant on the energy fluence rate and was DCMU sensitive, suggesting that photosynthetic electron transport was required for the light-dependant increase in activity. However, no increase of asparaginase activity was observed *in vitro* when extracts (obtained from the end of the dark period) were incubated with reduced ferredoxin, NADPH, or NADH, yet these compounds stabilized activity in the presence of asparagine; ATP inhibited asparaginase activity. The relative molecular mass of asparaginase, harvested at the end of the dark period, or during the light was the same (Mr. 68,000), and the enzymes co-eluted upon DEAE cellulose chromatography. The interaction between asparaginase activity

and ions was complex. Greater activity was noted if asparaginase was extracted in the presence of Ca^{2+} , however, no increase in activity was detected if Ca^{2+} was added to extracts; calmodulin does not appear to play a role in the regulation of asparaginase activity. Feeding inhibitors of protein and mRNA synthesis to half expanded leaves in the light indicated that *de novo* synthesis of mRNA and protein are required for the increased asparaginase activity. The decrease of asparaginase activity in the dark was not critically examined but results are consistent with proteolytic inactivation of asparaginase.

Acknowledgements

Funding for this project was supplied by NSERC grants to Dr. K.W. Joy and Dr. R.J. Ireland, and an NSERC post-graduate scholarship as well as a teaching assistance award from Carleton University.

I would like to thank Dr. K.W. Joy for his patience throughout my stay in his lab, his thought provoking questions and comments that appeared at the right times, and his excellent suggestions and criticisms during the writing of this thesis. Many thanks are also extended to Dr. R.J. Ireland, who set the seed that sparked my inquiry into enzyme regulation, and for his support and suggestions over the course of the study. Oh, and thanks K.W.J. and R.J.I. for the encouragement and financial aid in support of attending conferences across Canada and the US; these meetings were a great way to meet people, and they also opened my eyes to many aspects of plant physiology. I appreciate the useful suggestions from Dr. J. Sinclair (and the squash games!) on the work regarding photosynthesis, and thanks to Cathy Shay and Chris Mattson for their help in amino acid analysis. Many thanks to Louis Vézina and Dave Taylor for the 'think-tank' sessions, however infrequent, but they were always a joy.

Many heart-felt thanks go to Jill, who sat and listened to idea, after experiment, after result for too many nights; she's one ornithologist who understands nitrogen flow from N_2 to amino acid, and electron flow from water to NADPH! Also, thanks Jill for the proofing and typing in the crunch.

Last, but far from least, my love to little Sarah who placed this whole project into perspective.

TABLE OF CONTENTS

	Page
ACCEPTANCE SHEET	ii
ABSTRACT	iii
ACKNOWLEDGEMENTS	v
TABLE OF CONTENTS	vi
LIST OF TABLES	x
LIST OF FIGURES	xii
LIST OF ABBREVIATIONS	xv
 INTRODUCTION	
Asparagine as a storage and transport compound	1
Synthesis of asparagine	7
Metabolism and utilization of asparagine	11
Deamidation and transamination of asparagine	14
Support for asparagine metabolism in leaves: ¹⁵ N experiments	22
Asparagine and photorespiration	24
Properties of plant asparaginases	27
Microbial asparaginase and its regulation	28
Regulation of enzymes by light	32
 MATERIALS AND METHODS	46
Chemicals	47
Methods	
1) Enzyme extractions	47
Asparaginase	48
Asparagine aminotransferase	49
Glutamate dehydrogenase	50
Glutamine synthetase	50
Glutaminase	52
2) Analysis of aspartate or glutamate	
a) Amino acid analysis (Beckman analyzer)	53
b) Amino acid analysis by HPLC	53
c) Enzymatic analysis of glutamate	54
3) Analysis of ammonia	
a) Nessler's reagent	54
b) Microdiffusion of ammonia	55
4) Protein determinations	58
5) Analysis of leaf amino acid levels by HPLC	58

TABLE OF CONTENTS (continued)

Page

MATERIALS AND METHODS

Methods.

6) Analysis of xylem sap.	
a) Sap collection	59
b) Amino acid analysis of root exudates by HPLC	59
c) NO_3^- analysis of root exudates	59
7) Column chromatography.	
a) Ion exchanges	60
b) Gel permeation chromatography	61
8) Feeding experiments	61
9) Estimation of photosynthetic activity	
a) DCPIP reduction	62
b) O_2 evolution from chloroplast membranes	63
c) O_2 evolution from leaf pieces	63
d) <i>in vivo</i> fluorescence determination	64
10) Analysis of radioactivity in leaf homogenates	65

RESULTS 67

I. Activity of asparaginase from developing leaves	
1. Plant and leaf development	67
2. Asparaginase extractions and stability	70
3. Asparaginase inactivation	73
4. Diurnal variation of asparaginase activity in young leaves	76
5. Diurnal variation of amino acid and ammonia levels in young leaves	81
6. Diurnal changes of related enzymes of nitrogen metabolism	81
7. Asparaginase activity and leaf age	86
8. Asparaginase, leaf amino acid levels, xylem exudates and plant age	91
9. Asparaginase activity in detached leaves from soil grown plants	107
10. Asparaginase activity in detached leaves from hydroponic plants	109
11. Asparaginase activity: feeding experiments with MSO and AOA	111

TABLE OF CONTENTS (continued)

	Page
RESULTS	
II. Light and Asparaginase activity	
1. Asparaginase activity and extended dark/light periods	124
2. Asparaginase activity and energy fluence rates	124
3. Asparaginase activity and photosynthetic inhibitors	132
III. Studies on the mechanism of the light-dependent increase in asparaginase activity	
1. Is there a change in enzyme kinetics associated with increased asparaginase activity?	146
2. Asparaginase activity and products of the light reactions	147
3. Asparaginase activity, pH and Mg^{2+}	163
4. Asparaginase activity and K^+ ions	169
5. Asparaginase activity and some other ions	171
6. Asparaginase, Ca^{2+} and calmodulin	174
7. Asparaginase and phytochrome	183
8. Asparaginase and proteolysis	186
9. Is there a soluble factor affecting asparaginase activity?	190
10. Asparaginase, gel filtration and ion exchange chromatography	192
11. Asparaginase: membrane bound versus free enzyme	201
12. Asparaginase and protein synthesis	202
DISCUSSION	211
NH_3 determination by microdiffusion	211
Properties of asparaginase from developing leaves	214
Diurnal variation of asparaginase activity: leaf and plant age	220
Effects of light and dark on asparaginase activity	222
Possible mechanisms for regulation of asparaginase activity	228
Other <i>in vivo</i> factors influencing asparaginase	237
Asparaginase and NH_3 metabolism	239
Why does asparaginase activity vary diurnally?	244
Conclusions	246
LITERATURE CITED	249

TABLE OF CONTENTS (continued)

	Page
APPENDIX I: Hoaglands nutrient solution	283
APPENDIX II: Reversible inhibition of photosynthesis by DCMU	284

Table	LIST OF TABLES Description	Page
1	Changes in asparaginase activity in young and half expanded leaves during plant development	95
2	Amino acid levels in stage 3 leaves	98/99
3	Amino acid levels in half expanded leaves	100/101
4	Amino acid and nitrate levels in xylem exudates	105/106
5	Asparaginase activity in detached soil grown leaves	108
6	Analysis of xylem sap from soil and hydroponic plants	110
7	Effect of feeding xylem sap on asparaginase activity in detached leaves	112
8	Asparaginase activity in detached hydroponic and soil grown plants	114
9	Ammonia accumulation in leaves and effects of inhibitors	117
10	Effects of MSO or asparagine on asparaginase activity in detached leaves during the light period	122
11	Effect of DCMU and sucrose on asparaginase activity in detached leaves	145
12	Effect of photosynthetic products on asparaginase activity	153
13	Effect of incubating asparaginase with compounds of interest but without asparagine	155
14	Incubation of S300 - asparaginase with products of the light reactions	161
15	Diurnal variation of asparaginase activity in the absence of K^+ ions	170

LIST OF TABLES (continued)

Table	Description	Page
16	The effects of ions on asparaginase activity	175
17	Is asparaginase activity mediated by phytochrome?	187
18	Effect of PMSF and iodoacetic acid on asparaginase	191
19	Effect of coharvesting on asparaginase activity	193
20	Is asparaginase membrane bound?	203
21	Effect of protein synthesis inhibitors on asparaginase activity	207

LIST OF FIGURES

Figure	Description	Page
1	Ammonia nitrogen standard curve	57
2	Stages and age of developing pea leaves	69
3	Stability of asparaginase during storage at 4° C	72
4	Stability of asparaginase at 34° C	75
5	Effect of asparagine, aspartate, and ammonia on the stability of asparaginase	78
6	Diurnal variation of asparaginase activity in young leaves	80
7	Diurnal changes of several amino acids in young leaves	83
8	Diurnal changes in glutamine synthetase activity and ammonia levels in young leaves	85
9	Diurnal changes in glutamate dehydrogenase and glutaminase activities in young leaves	88
10	Diurnal variation of asparaginase during leaf development	90
11	Diurnal variation of asparaginase activity in half expanded leaves	93
12	Amino acid levels in successive young and half expanded leaves during plant development	97
13	Amino acid and nitrate levels in xylem exudates during plant development	104
14	Effects of MSO on asparaginase activity, glutamine synthetase activity, and ammonia levels in detached leaves	120
15	Effects of prolonged light or dark periods on asparaginase activity	126

LIST OF FIGURES (continued)

Figure	Description	Page
16	Effect of energy fluence rate on asparaginase activity	129
17	Time course of asparaginase activity at two different energy fluence rates	131
18	Effect of DCMU and atrazine on <i>in vitro</i> photosynthetic activity	134
19	Effect of DCMU or atrazine on the fluorescence induction curve	137
20	Effect of DCMU on O ₂ evolution from leaf pieces and <i>in vivo</i> fluorescence	139
21	Effect of DCMU or atrazine on asparaginase activity	141
22	Effect of DCMU or atrazine on asparaginase and photosynthetic activities	143
23	Michaelis - Menten kinetics of asparaginase	149
24	Michaelis - Menten kinetics of asparaginase. Lineweaver - Burk plot	151
25	Inhibition of asparaginase activity by MgATP	158
26	Inhibition of asparaginase by MgATP. Lineweaver - Burk plot	160
27	Asparaginase activity and pH	166
28	Effect of MgCl ₂ on asparaginase activity	168
29	Effect of Na ⁺ and K ⁺ on asparaginase activity	173

LIST OF FIGURES (continued)

Figure	Description	Page
30	Effect of Ca^{2+} on asparaginase activity	177
31	Asparaginase activity: extractions in CaCl_2	179
32	Asparaginase activity: extraction in CaCl_2 at the end of the dark or during the light periods.	182
33	Inhibitors of calmodulin action on asparaginase activity	185
34	Effects of proteinases on asparaginase activity	189
35	Gel permeation chromatography of asparaginase	196
36	Relative molecular mass of asparaginase	198
37	DEAE cellulose chromatography of asparaginase	200
38	Effects of inhibitors of protein synthesis on asparaginase activity	206

LIST OF ABBREVIATIONS

A _λ	absorbance (optical density) at specified wavelength, λ
AAN	amino acetonitrile
AEC	adenylate energy charge
AOA	aminooxyacetate
DCMU	3-(3',4'-dichlorophenyl)-1,1-dimethylurea
DCPIP	dichlorophenol indophenol
DMSO	dimethyl sulfoxide
DONV	5-diazo-4-oxo-L-norvaline
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EPPS	N-(2-hydroxyethyl) piperazine-N'-3-propane sulfonic acid
FBPase	fructose 1,6-bisphosphatase
GAPD	glyceraldehyde 3-phosphate dehydrogenase
GDH	glutamate dehydrogenase
GOGAT	glutamine:oxoglutarate aminotransferase
G6PD	glucose 6-phosphate dehydrogenase
GS ₁	glutamine synthetase - cytosolic isoform
GS ₂	glutamine synthetase - chloroplastic isoform
GSH	reduced glutathione
GSSH	oxidized glutathione
HEPES	N-2-hydroxyethylpiperazine-N'-2 ethane sulfonic acid
Hoba	4 hydroxy 2-oxobutyric acid
HPMS	4-hydroxy-2-pyridine methanesulfonic acid
HPLC	high performance liquid chromatography
Hse	homoserine
INH	isonicotinyl hydrazide
K _i	dissociation constant for an enzyme inhibitor complex
K _{i0}	K _i of enzyme inhibitor complex
K _{i0s}	K _i of enzyme and substrate inhibitor complex
K _m	Michaelis constant (substrate concentration giving 1/2 V _{max})
LEM	light effect mediator
LHCP	light-harvesting chlorophyll <i>a/b</i> protein
MDH	malate dehydrogenase
MES	4-morpholine ethane sulfonic acid
MOPS	4-morpholine propane sulfonic acid
Mr	relative molecular mass

LIST OF ABBREVIATIONS

MSO	methionine sulfoximine
Na ₂ SO ₄	sodium dithionite
NCR	nitrogen catabolite repression
NH ₃	ammonia (predominantly ammonium at physiological pH)
NiR	nitrite reductase
NR	nitrate reductase
PAL	phenylalanine ammonia lyase
PAL-IS	phenylalanine ammonia lyase-inactivating system
PAR	photosynthetically active radiation
PDC	pyruvate dehydrogenase complex
PDK	pyruvate, Pi dikinase
PEP	phosphoenolpyruvate
PGK	3-phosphoglycerate kinase
PMSF	phenylmethyl sulphonyl fluoride
PQ	plastoquinone
PRK	ribulose 5-phosphate kinase
Q	primary electron acceptor
QNOR	quinine:NAD ⁺ oxidoreductase
Rubisco	ribulose biphosphate carboxylase oxygenase
SBPase	sedoheptulose 1,7-bisphosphatase
Tricine	N-Tris (hydroxymethyl) methylglycine
Triton	octyl phenoxy polyethoxy ethanol
Tris	tris-(hydroxymethyl)-aminomethane
V _{max}	maximum rate of reaction

"But everybody knows that enzymes are trained from birth never to give valuable information once they are outside the cell."

D. Recalain

Up the carbon path,

New Scientist, 1968.

"I am beginning to believe that nothing can ever be proved. These are honest hypothesis which take the facts into account: but I sense so definitely that they come from me, and they are simply a way of unifying my own knowledge. Slow, lazy, sulky, the facts adapt themselves to the rigour of the order I wish to give them; but it remains outside of them. I have the feeling of doing a work of pure imagination."

J-P. Sartre

nausea

Introduction

An actively developing leaf requires a source of energy, carbon, nitrogen, water, and essential elements for growth. In addition to these factors, a leaf also requires internal control systems that regulate different metabolic processes during growth and development. Energy and carbon are initially supplied from carbohydrate reserves, or in established plants, from sunlight through the processes of photosynthesis. Since atmospheric nitrogen is not assimilated within leaves, nitrogen is obtained from the soil by the root, and is transported through the plant in the xylem or phloem. Water and essential elements are also derived from the soil by the roots and arrive at the leaf through the xylem or phloem. Systems that regulate metabolic processes in a leaf are controlled either by light directly, or by products arising from active metabolism.

This study considers the relationship between light and asparaginase (L-asparagine amido hydrolase EC 3.5.1.1), an enzyme that metabolizes asparagine (a protein amino acid and major transport compound), in developing leaves of the pea plant, *Pisum sativum*, and examines possible regulatory mechanisms involved in modifying asparaginase activity.

Asparagine as a storage and transport compound

Asparagine is a commonly translocated form of nitrogen in many plants. This is in part due to the relatively low carbon to nitrogen (C:N) ratio for asparagine, a 4 carbon compound with 2 nitrogens thus the C:N ratio is 2 (Pate 1973). Other recognized nitrogen transport compounds

have C:N ratios that are higher (for example glutamine, 2.5), equal (e.g. citrulline, 2), or lower (e.g. the urides allantoin and allantoic acid both have a C:N ratio of 1) than that of asparagine. Asparagine, glutamine and the nonprotein amino acid homoserine are the main nitrogen constituents in the xylem of nodulated or nonnodulated peas (Pate 1980), the plant used in the present study.

Another reason that asparagine is suitable as a nitrogen transport compound in many plants, is that it is relatively mobile in the pH range found in transport streams of plants; some compounds are transported greater distances, while others do not transport as well as asparagine. The pH of xylem fluid is acidic (pH 5.4 to 6.3) while phloem exudate is more alkaline (pH 7.4 to 8.6, Pate 1980). Under these conditions anionic compounds travel greater distances than neutral or cationic compounds (McNeil et al. 1979). For example aspartic and glutamic (and allantoic, Pate 1980) acids are negatively charged and are transported through the xylem without lateral abstraction usually arriving and undergoing extensive metabolism in actively transpiring leaves (Urquhart and Joy 1982). However, the cationic protein amino acid arginine (a transport compound in perennials, Lea and Mifflin 1980, with a low C:N ratio of 1.5), is positively charged in the xylem (Larson 1980) and is therefore easily abstracted. Allantoin, and the amides asparagine and glutamine are neutral and are transported greater distances than arginine within the xylem before abstraction (McNeil et al. 1979). The differences in mobility between neutral and anionic compounds coincides with the differences in the metabolism of these compounds in leaves. Aspartic and glutamic acids are routinely turned over in leaves and are involved in many metabolic processes, whereas asparagine is more selectively

metabolized supplying nitrogen in growing regions (Pate 1980, 1983a).

The reduction of nitrate (NO_3^-) to the level of NH_3 in pea plants may take place in the root, or NO_3^- may be transported through the xylem for reduction in the leaf, in either case the reactions are catalyzed by nitrate reductase and nitrite reductase. When inorganic nitrogen levels are low in the soil, atmospheric nitrogen (N_2) may be reduced to the level of NH_3 within nodules of the pea plant in a reaction catalyzed by nitrogenase (e.g. Pate 1980). Ammonia, arising from nitrite reductase or nitrogenase activity within the root, is assimilated in the presence of glutamate to form glutamine (by glutamine synthetase) and this product may then be exported from the root since it is a nitrogen transport compound found in the xylem of many plants including the pea. On the other hand, glutamine may be used as a substrate, along with aspartate, in the synthesis of asparagine, in a reaction catalyzed by asparagine synthetase (Mifflin and Lea 1980). The newly synthesized asparagine is then exported from the root to developing regions of the plant requiring nitrogen for growth.

In young developing pea leaves asparagine has been found to be actively metabolized, supplying nitrogen for amino acid and protein synthesis. However, in mature leaves that no longer require nitrogen for growth, asparagine is not readily metabolized and is reexported (in the phloem) from the leaf to regions of active growth such as developing leaves and seeds (McNeill et al. 1979, Urquhart and Joy 1982).

Asparagine is the major nitrogen compound detected in the phloem of several legumes and concentrations of 30 mM have been reported (Atkins et al. 1975). The process of reexport of asparagine from the mature leaf

to the phloem appears to involve xylem to phloem transfer with little metabolism of asparagine in the mature leaf (Sharkey and Pate 1975, Pate 1980, 1983a). This process is quite selective since many of the components of the xylem are not detected in the phloem. Solutes present in the xylem such as aspartic and glutamic acids, and NO_3^- (and other ions e.g. Ca^{2+} , Zn^{2+} and Mn^{2+}) are not generally found in the phloem, while high levels of carbohydrate (and other compounds such as growth-regulating substances), not present in the xylem, are detected in phloem exudates (Pate 1983a).

Two other factors also point to asparagine as a good nitrogen—transport compound within plants. One is its stability in solution if compared with glutamine (Larson 1980), or the urides (Pate 1983b), and the other is its limited involvement in metabolic processes.

Asparagine's limited involvement in cellular processes has lead to much confusion regarding its role in plants since it was first isolated in 1806. To emphasize this point three examples are presented: 1) in 1968 a report concluded that asparagine was to be considered a "dead-end" metabolite, used "only for protein synthesis on a quantitatively significant scale" and its role as a nitrogen transport compound was questioned (Lees et al. 1968), 2) more recently Cooney et al. (1980), in a study on asparagine metabolism in *Asparagus officinalis* seedlings, also suggested that asparagine was a dead-end metabolite and that "despite years of phytochemical effort, the exact role for this huge storage depot remains to be elucidated.", and 3) a study examining the metabolism of serine and glycine in photosynthetic and nonphotosynthetic tissues of *Pisum sativum* by Walton and Woolhouse (1986) concluded that "the physiological significance of these

[transamination] reactions, especially with asparagine, must remain questionable."

Asparagine was first isolated from the juice of asparagus in 1806 by Vauquelin and Robiquet (for a translation of their paper see Cooney et al. 1980). After the discovery of asparagine in *Asparagus sativus*, it was detected in vetch extracts by Pira in 1844, who also observed that the levels of asparagine decreased with plant age (Chibnall 1939). Shortly thereafter the relationship between light and asparagine became apparent, when in 1858 Sullivan noted that etiolated vetch seedlings accumulated high levels of asparagine, and the levels decreased rapidly when the seedlings were exposed to light (Chibnall 1939). A decade later Boussingault proposed a hypothesis to account for the change in asparagine concentrations in plants that took place between the light and dark (quoted from Chibnall 1939):

"Just as part of the protein ingested by an animal (he argued) is modified during respiration to urea, which is excreted, so also in the darkened plant there is a similar respiratory combustion of protein to give, not urea, for the plant cannot excrete, but asparagine, which remains dissolved in the sap and can be again utilized by the plant under the action of light. Asparagine can thus be produced in the roots, stems and leaves, but only in the dark, and it will accumulate only as long as the light necessary for its modification is withheld."

The relationship between the increase of asparagine in the dark and its decrease in the light has been reported many times in the literature (e.g. Steward and Durzan 1965, Carr and Paté 1967, Lea and Fowden 1975a, Joy et al. 1983, Sieciechowicz et al. 1985).

Prianischnikow, in 1922, was the first to observe that asparagine

increased in light in the presence of a carbohydrate (sucrose) and NH_3 and he suggested that the accumulation of asparagine in the presence of NH_3 may be a mechanism of detoxification (Chibnall 1939). The synthesis of asparagine was investigated in more detail by Mothes in 1933 who found that asparagine levels increased in leaves infiltrated with ammonium aspartate and several organic acids (Chibnall 1939). The role of asparagine as a detoxification product, acting as a nitrogen storage compound synthesized under high NH_3 conditions, has been firmly established (Givan 1979).

The amides, asparagine and glutamine, are important products of protein degradation, synthesized upon hydrolysis of storage proteins during seed germination (Dilworth and Dure 1978, De Ruiter and Kolloffel 1983, Lea and Joy 1983), or the breakdown of proteins during leaf senescence, and subsequently transported to developing leaves or fruits through the phloem (Carr and Pate 1965). The involvement of asparagine as a product of protein degradation during seed germination was noted by Schulze and Castoro in 1903 (Chibnall 1939), and the synthesis of asparagine during leaf senescence was first reported in 1897 by Miyachi (Lea and Milfin 1980). At the time that Chibnall wrote his review of protein metabolism in plants (1939) the role of asparagine in plants as a nitrogen transport compound, as a detoxification product, and as a compound that varied in concentration in the light and dark was known, yet the mechanisms accounting for the synthesis or degradation of asparagine were still uncertain. The lack of certainty with respect to asparagine synthesis or degradation still remains today.

Synthesis of asparagine

Three routes have been proposed to account for asparagine synthesis. One involves the synthesis of asparagine from 2-oxosuccinamic acid (the β -amide of oxaloacetic acid), and a suitable amino donor in a reverse reaction of asparagine:oxoacid transaminase (EC 2.6.1.14). This reaction has been detected in rat liver extracts, free from asparaginase but not α -amidase activities (see below) and therefore the presence of high 2-oxosuccinamic concentrations (0.1 M) were required for asparagine synthesis (Meister and Fraser 1954). The preferred amino donors for asparagine synthesis were α -aminobutyric acid, alanine, glutamine, and norvaline, although several other amino acids participated in the reaction (asparagine was also formed from 2-oxosuccinamic acid with a glutamine transaminase preparation from liver or kidney in a reaction specific for glutamine as the amino donor, Meister and Fraser 1954). This mechanism for asparagine synthesis was also reported by Mardashev and co-workers (Lerman and Mardashev 1960, see also Webster and Varner 1955) in crude rat liver and rabbit liver or kidney extracts. However, synthesis of asparagine by this route has been demonstrated only at low levels in leaves of *Pisum sativum* (20 fold less activity than the asparagine utilizing direction of the reaction, Ireland and Joy 1983) and is of questionable physiological importance in plants.

A second pathway of asparagine synthesis involves the incorporation of cyanide and cysteine into β -cyanoalanine (catalyzed by β -cyanoalanine synthase, EC 4.4.1.9) which is then hydrolyzed (β -cyanogalanine hydrolase, EC 4.2.1.65), producing asparagine. This pathway of asparagine synthesis has been demonstrated in a variety of plants. For example, both enzymes have been isolated in *Lupinus*

angustifolius seedlings and cotyledons (Hendrickson and Conn 1969, Castric et al. 1972) and *Asparagus officinalis* seedlings (Cooney et al. 1980), and an active β -cyanoalanine synthase (10 fold higher activity than asparagine synthetase activity) has been demonstrated in root tips of *Zea mays* (Stulen et al. 1979). The major source of cyanide is thought to be from cyanogenic glycosides which only occur in a limited range of plants (Ting and Zscheche 1970, Streeter 1973). Therefore, the β -cyanoalanine pathway as a sole route of asparagine synthesis has been questioned and is considered of importance only as a cyanide detoxifying mechanism (e.g. Lea and Mifflin 1980), however this has been challenged (Wurtele et al. 1984). Another possible source of cyanide in plants was proposed by Solomonson and Spehar (1977) in a study on the regulation of NO_3^- reductase activity in *Chorella vulgaris*. They observed that hydroxylamine, an intermediate of NO_2^- reduction, and glycolate, an intermediate of the photorespiratory pathway, produced cyanide in the presence of a soluble extract from *Chorella*. This reaction has also been confirmed in leaves of spinach, maize, and barley, and was dependant upon ADP, and Mn^{2+} for optimal activity (Huckelsby et al. 1982). Recently, cyanide synthesis has been observed during ethylene biosynthesis (Peiser et al. 1983, Wurtele et al. 1984), again rekindling the debate regarding the importance of the β -cyanoalanine pathway in asparagine synthesis.

The third route leading to asparagine synthesis is mediated by asparagine synthetase (EC 6.3.5.4), and involves an ATP-dependant amide transfer from glutamine (and in some cases NH_3 , see below) to aspartate (in the presence of Mg^{2+}) producing asparagine, glutamate, AMP and PPi .

Asparagine synthetase from all plant sources so far examined is very unstable, and required the addition of protectants (DTT, mercaptoethanol, and glycerol) in order to detect activity. Other confounding factors that affected detection of asparagine synthetase activity in crude preparations include the presence of inhibitors (Kern and Chrispeels 1978, Joy et al 1983, Joy 1985), asparaginase activity (especially in nodule preparations, e.g. Huber and Streeter 1985), glutaminase activity (Rognes 1980), and in the case of NH_3 as substrate, glutamine synthetase activity (Lea and Mifflin 1980).

The first report of asparagine synthesis in plants (Webster and Varner, 1955) indicated that NH_3 was incorporated into asparagine in an ATP-dependant reaction analogous to glutamine synthetase (although glutamine did not participate in the reaction leading to asparagine synthesis). This conclusion was consistent with feeding experiments demonstrating that asparagine concentrations increased in leaf discs infiltrated with aspartate, NH_3 and ATP (e.g. Yamamoto 1955). However, the reaction mixture used by Webster and Varner (1955) has been criticized for several reasons (Streeter 1973, Lea and Fowden 1975a), one being the high concentrations of aspartate and hydroxylamine required to detect asparagine synthesis. Recent reports have indicated that glutamine, and not NH_3 , is the preferred nitrogen donor for asparagine synthetase extracted from germinating cotyledons (e.g. *Glycine max* Streeter 1973, *Lupinus luteus* Rognes 1975, 1980, and *Lupinus albus* Lea and Fowden 1975b, *Gyssypium hirsutum* Dilworth and Dure 1978), root nodules (*Lupinus angustifolius*, Scott 1976, *Glycine max*, Huber and Streeter 1985), and mouse leukemia

cells (Horowitz and Meister 1972). Stulen et al. (1979) reported that asparagine synthetase extracted from mature *Zea mays* roots displayed only a marginal preference for glutamine over NH_3 . From this and other observations Oaks and Böss (1983) suggested that asparagine synthetase from maize root may be different from legume cotyledon and nodule enzymes.

The occurrence of asparagine synthetase activity in different plant organs is not well documented. The absence of asparagine synthetase in *Asparagus officinalis* seedlings (isolated without added protectants) led Cooney et al. (1980) to conclude that the β -cyanoalanine pathway was responsible for asparagine synthesis, yet β -cyanoalanine hydrolase, the enzyme leading to asparagine synthesis from β -cyanoalanine, was present only at very low levels. Asparagine synthetase activity was also absent from leaves of *Pisum sativum* (extracted with various protectants, Joy et al. 1983), even though detached shoots were able to synthesize asparagine. Bauer et al. (1977a) demonstrated an increase and decrease of ^{15}N in asparagine in detached pea leaves fed $^{15}\text{NO}_3^-$, followed by $^{14}\text{NO}_3^-$, which indicated that asparagine was synthesized and turning over in leaf tissues.

Several reports, from plant and animal sources, have indicated that asparagine synthetase is sensitive to several cations and anions. For example, asparagine synthetase, and associated glutaminase activities were observed to be Cl^- stimulated in mouse leukemia cells (Horowitz and Meister 1972) and *Lupinus luteus* cotyledon extracts (Rognes 1980), and a strong inhibition of lupin cotyledon asparagine synthetase has been reported in the presence of Ca^{2+} (Rognes 1975, Joy 1985).

The occurrence of a heat-stable, dialyzable inhibitor (possibly Ca^{2+} , Joy 1985) of lupin-cotyledon asparagine synthetase was observed in pea leaf homogenates (Joy et al. 1983), and these authors cautioned against dismissing asparagine synthetase as a plausible route for asparagine synthesis if activity is not detected in extracts. For example, homogenates of asparagus seedlings, reported to lack any asparagine synthetase (Cooney et al. 1980), were observed to strongly inhibit asparagine synthetase activity obtained from lupin cotyledons (Joy et al. 1983). The results from pea and asparagus inhibitor experiments suggest that detection of asparagine synthetase activity in leaf or other tissues may be impeded by inhibitors complexing with the enzyme during extraction. The presence of a heat-labile, nondialyzable inhibitor of asparagine synthetase (not present in 3 day old cotyledons), was observed in developing *Vigna radiata* seedlings, possibly accounting for reduced asparagine synthetase activity noted in older seedlings (Kern and Chrispeels 1978).

Metabolism and utilization of asparagine

Asparagine has been proposed to be metabolized by four routes (see diagram below, although the first two of these routes have been questioned in the literature, since the enzymes responsible for the reactions have not been isolated). One involves the transfer of the amide group of asparagine to glutamate to form glutamine through the reversal of asparagine synthetase (1) as concluded by Baurerova and Shorm in 1959 in study on rape seedlings (Lerman and Mardashev 1960). This route of asparagine metabolism was also supported by ^{15}N experiments in *Pisum sativum* leaves, demonstrating a rapid incorporation of ^{15}N ,

from ^{15}N (amide)asparagine, into glutamine (Bauer et al, 1977b), however rapid transfer of label from ^{15}N (amide)asparagine to glutamine was not observed by Atkins et al. (1980). A second route involves the reductive transamidation of asparagine with 2-oxoglutarate (2), in an analogous manner to glutamate synthase (Dougall 1974, Fowler et al. 1974). It has been suggested that contamination of asparagine with aspartate (readily transaminating to oxaloacetate which would then be reduced by malate dehydrogenase) could account for the oxidation of NAD(P)H observed in plant extracts by Dougall or Fowler et al. (Mifflin and Lea 1975).

Therefore, as a precautionary step, asparagine should be passed through Dowex 1 (at pH 6.8 to 7.0) to remove any contaminating aspartate before use in assays (Prusiner and Milner 1970, Mifflin and Lea 1975).

The two other routes of asparagine metabolism have been confirmed by identification of the presence of the appropriate enzyme. One route involves hydrolysis of asparagine, catalyzed by asparaginase to produce

aspartate and NH_3 (3). The second route involves the transamination of asparagine (in the presence of an oxo acid) producing 2-oxosuccinamic acid and an amino acid (4).

Asparaginase has attracted a lot of attention from the medical field after the discovery that the antineoplastic agent in guinea pig serum, administered for the treatment of acute lymphocytic leukemia, was asparaginase (Broome 1961). This finding was not of clinical importance until Mashburn and Wriston (1964) observed that *Escherichia coli* asparaginase also possessed antileukemic activity, thereby accessing an adequate supply for medical application (Wriston and Yellin 1973). Two asparaginases were isolated from *E. coli*, EC-1 and EC-2, and only EC-2 had antitumour activity. However, significant toxicity has been observed during asparaginase therapy, possibly a result of the depletion of blood glutamine levels (Durden et al. 1983, Kafkewitz and Bendich 1983), and this has been attributed to the associated glutaminase activity of EC-2 asparaginase (Prager et al. 1982). For this and other reasons (Wriston and Yellin 1973) an extensive search for alternate sources of asparaginase, without glutaminase activity, was carried out (see Imada et al. 1973).

To date two plant sources of asparaginase, without glutaminase activity, have been tested for antitumour activity with little success. Asparaginase purified from *Chlamydomonas* satisfied many of the requirements for antitumour activity although the K_m for asparagine was high and therefore showed limited antitumour activity (Paul 1982). Asparaginase purified from *Lupinus polyphyllus* also failed to demonstrate appreciable antitumour activity in the antilymphoma assay

(Miflin, personal communication) most probably due to its low affinity towards asparagine.

Deamidation and transamination of asparagine

The hydrolysis of asparagine by asparaginase, producing aspartate and NH_3 , was first detected in homogenates from *Penicillium camemberti* by Dox in 1909 (Imada et al. 1973). In plants, asparaginase was initially detected in roots of germinating barley seeds by Chibnall and Grover in 1926 (Chibnall 1939), although the activity of this preparation was quite low (Kretovich 1958, Lees and Blakeney 1970). Other early reports of asparaginase, obtained from *Aspergillus niger*, include Bach in 1928, and Schmalfluss and Mothes in 1930 (Wriston and Yellin 1973).

In 1946 Greenstein and Carter noted weak deamidation of asparagine, and glutamine (detected by NH_3 production) in aqueous rat liver extracts. The deamidation of asparagine or glutamine increased when pyruvate was added to assay mixtures. These results were interpreted to involve two separate reactions: 1) the condensation of asparagine (or glutamine) and pyruvate to form a dehydropeptide, and 2) the cleavage of the dehydropeptide (by a dehydropeptidase) to form pyruvate and NH_3 . The net result of both reactions was deamidation of asparagine (or glutamine), along with the recovery of pyruvate. Further work demonstrated that two asparaginases (or glutaminases) were present, one was heat-labile, and called asparaginase I, while the other, called asparaginase II, was heat-stable and activated in the presence pyruvate (Errera 1949, Greenstein and Price 1949). Several years later, Meister

and co-workers demonstrated that asparaginase II activity (i.e. pyruvate stimulated asparaginase activity) was due to the combined action of asparagine transamination, producing 2-oxosuccinamic acid, and the deamidation of 2-oxosuccinamic acid, by α -amidase, to yield oxaloacetic acid and NH_3 (Meister and Tice 1950, Meister et al. 1952, Meister 1953, Meister 1954, Meister and Fraser 1954, Meister et al. 1955). Meister (1953) also demonstrated that spinach and lettuce leaves contained α -amidase activities, capable of hydrolyzing oxosuccinamic acid (and oxoglutaramic acid, the β -amide of oxoglutarate), which suggested that the associated transaminase activity would be present.

Asparagine transaminase was initially detected in plants by Wilson et al. (1954), Yamamoto (1955), and later by Gamborg (1965) and Cincerova (1969). Yamamoto (1955) and Cincerova (1969) reported that asparagine transamination occurred in the presence of oxoglutarate, an observation which has not been supported (Ireland and Joy 1983a). Cincerova (1969) noted that a considerable amount of aspartate was present in assay solutions and suggested that the extracts contained asparaginase, in addition to asparagine transaminase, activity. However, the suggestion that asparaginase activity was present in their extracts is questionable when one considers the difficulties other workers have had in attempting to demonstrate asparaginase activity in vegetative structures (see below). A more plausible explanation of their results may be that aspartate was not removed from asparagine solutions used in their assays (e.g. by Dowex 1), and aspartate is known to readily transaminate in the presence of oxoglutarate and other oxo-acids (Givan 1980).

In 1954 Kretovitch and workers obtained extracts from lupin,

pumpkin, and corn which contained amidases capable of splitting asparagine and glutamine to form ammonia (Kretovitch 1958). At about this time, Webster and Varner (1955) reported on the synthesis of asparagine from aspartate and NH_3 . However, work a decade later was unable to confirm either the synthesis, or the hydrolysis of asparagine in wheat (Lees et al. 1968) or cotton seedlings (Ting and Zschoche 1970) and cast doubt upon the role and metabolism of asparagine in plants.

Several years after the observation that asparagine hydrolysis could not be detected in young seedlings, Lees and Blakeney (1970) reported on the presence of asparaginase in extracts obtained from mature *Lupinus luteus* (in potassium phosphate buffer). They examined 10 week old nodulated and nonnodulated (grown on NH_3 or NO_3^-) plants and observed that high asparaginase activity (specific activity not mentioned) was found in both the plant and bacteroid fractions of nodules and in root extracts, while only low levels of activity occurred in the leaves (age unspecified). They also pointed out that roots contained little free amide while the bulk of the asparagine was found in the leaves, and they suggested that this was consistent with the data on the distribution of asparaginase. Streeter (1977) concluded that asparaginase activity in the plant fraction of nodules, prepared from 30 to 70 day old *Glycine max*, was probably a contaminant of bacteroid-asparaginase during extract preparation. However, Scott et al. (1976), analysing NH_3 assimilation in nodulated *Lupinus angustifolius*, also detected asparaginase in the plant fraction of nodules (extracted in potassium phosphate buffer), and noted that the activity decreased during the first two weeks after nodulation. They postulated that the asparaginase

detected in the plant fraction of the nodule hydrolysed incoming asparagine, synthesized from cotyledonary reserves, and thereby supplied nitrogen and C required for nodule growth prior to the establishment of active nitrogen fixation (suggesting that asparaginase obtained from the plant fraction was different from nodule asparaginase).

Lea and Fowden (1975a) criticised the work of Lees et al. (1968) and Ting and Zchoche (1970) on asparagine metabolism in seedlings and suggested that a more likely site of asparagine metabolism would be one where nitrogen is required for growth, such as developing seeds undergoing active protein synthesis. For example, Lea et al. (1979) demonstrated that *Pisum sativum* cotyledons, maintained in sterile culture with asparagine as the sole nitrogen source, could support high rates of protein synthesis, and a more recent report by Skokut et al. (1982) suggested that asparagine was deamidated in cotyledons of *Glycine max* grown in culture with ^{15}N (amide)asparagine (detected by solid state [^{15}N]NMR). However, many studies have failed to demonstrate any asparaginase activity in developing seeds from a range of plants, such as *Pisum*, *Phaseolus*, *Vicia* (Lea et al. 1976, using Tris buffer), *Pisum* (Beevers and Storey 1976, using Tris-sodium phosphate buffer), or several *Lupinus* varieties (Lea et al. 1978, using Tris buffer). On the other hand, Atkins et al. (1975, phosphate buffer cation unspecified) detected asparaginase in developing seeds obtained from *Lupinus albus*.

The intermittent occurrence of asparaginase activity in plants was partially explained in a report by Sodek et al. (1980), who demonstrated that asparaginase, obtained from developing seeds of *Pisum sativum*, was a potassium-dependant enzyme. Maximum asparaginase activity in

crude extracts obtained in Tris HCL buffer was obtained when 20 to 50 mM KCl was added to the assay solution. Developing seeds from *Pisum arvense*, *Phaseolus multiflorus*, *Vicia faba*, *Lupinus albus*, *L. mutabilis*, *Zea mays*, and *Hordeum vulgare* all demonstrated a stimulation of asparaginase activity with potassium. Chang and Farnden (1981) reported the occurrence of two potassium-independent asparaginases in developing seeds of *Lupinus arboreus* and *Lupinus angustifolius*, although preparations of roots, leaves, developing nodules, and flower buds required potassium for activity. In another study, 54 *Lupinus* varieties (including several species) were tested with antiserum raised against a potassium-independent asparaginase from *Lupinus polyphyllus*, and 11 were found to have potassium independent asparaginases (Lea et al. 1984). Asparaginase obtained from *Chlamydomonas* was also found to be potassium-independent (Paul and Cooksey 1979, 1981a, 1981b, Paul 1982).

Prior to the observation for the potassium dependency of asparaginase, utilization of asparagine in developing seeds was understood in several species, although the sporadic occurrence of the enzyme was indeed baffling. In a comprehensive study on nitrogen utilization during seed development, Atkins et al. (1975) demonstrated that asparagine was metabolized in developing *Lupinus albus* seeds. They fed $^{14}\text{C}(\text{U})$, $^{15}\text{N}(\text{amide})$ asparagine via the transpiration stream to fruiting shoots and noted that the bulk of the label, as asparagine, was translocated to the seeds. Most of the ^{15}N in the endosperm fluid was recovered in NH_3 , glutamine, and alanine (trace amounts were detected in serine, β -aminobutyric acid, histidine, and arginine), while most of the ^{14}C was found in non-amino compounds (trace amounts noted in

glutamine, alanine, serine). As the seeds developed, both ^{15}N and ^{14}C were detected in amino acid constituents of seed storage proteins. Asparaginase activity, assayed in crude embryo-extracts, increased approximately 10-fold during seed development and reached maximum activity 6 to 7 weeks after anthesis (extracts were obtained in phosphate buffer, cation unspecified, although Lea et al., 1984, indicated that the cation was potassium). Asparaginase was present in trace amounts in the seed coat, and liquid endosperm, but the bulk of activity was present in the embryo. The increase in asparaginase activity during seed development also preceded the increase in embryo fresh weight, supporting the role of asparagine as a nitrogen transport compound, supplying nitrogen required for growth (Atkins et al. 1975).

Shortly thereafter, asparaginase was purified from maturing *Lupinus polyphyllus* seeds (in Tris buffer, Lea et al. 1978), and an apparent K_m for asparagine of 12.2 mM, and a relative molecular mass of 72,000 (determined via linear sucrose density gradient) were estimated. These authors also suggested that asparaginase was 1) a soluble enzyme, not associated with any organelle, 2) a dimer with subunits of similar molecular weights (based on SDS gel electrophoresis), and 3) that the subunits of asparaginase may undergo aggregation. No asparaginase activity was detected in pod, leaf (age unspecified), stem, or root tissues. A survey of the distribution of asparaginase in developing seeds of several *Lupinus* species indicated that asparaginase was found only in a limited number of species and no asparaginase activity was detected in maturing seeds from *Pisum sativum* (Lea et al. 1978). However in 1980 Murray and Kennedy reported on the presence of asparaginase activity in developing seeds from two varieties of *Pisum sativum*

(extracted in potassium phosphate buffer). Asparaginase activity decreased in the seedcoats from 15 to 27 days after anthesis, and increased in the cotyledon over the same time period. An increase of the protein levels in the cotyledon followed the rise in asparaginase activity by several days (for related enzymes of nitrogen metabolism in developing seeds see Beevers and Storey 1976, De Ruiter and Kolloffel 1982, Gomes and Sodek 1984).

In 1977 Streeter detected active transamination of asparagine in leaves of *Glycine max* while only marginal asparaginase activity was observed (age unspecified, extracted in Tricine buffer). Based upon the high specific activity of the transaminase (higher than the specific activity of asparaginase), he suggested that transamination was the major metabolic route for asparagine metabolism in leaves. Lloyd and Joy (1978) noted that after feeding ^{14}C -asparagine to leaves of *Pisum sativum* and *Glycine max*, label appeared in hydroxysuccinamic acid (see diagram end of next section). This compound was also formed when 2-oxosuccinamic acid was fed to leaves, and they suggested that following asparagine transamination, 2-oxosuccinamic acid was reduced to hydroxysuccinamic acid.

An extensive study by Cooney et al. (1980) on asparagine metabolism in *Asparagus officinalis* failed to demonstrate appreciable asparaginase activity in homogenates of 10 cm seedlings (obtained in Tris buffer without K^+), while 60 cm plants exhibited 3 to 5 fold more asparaginase activity, although the activity was still very low (2 nmol/hr./mg.protein). They also reported that asparagine aminotransferase was present at low levels in whole homogenates of *Asparagus officinalis* and suggested that the limited utilization of

asparagine by asparaginase or asparagine aminotransferase is consistent with the high concentrations of asparagine found in asparagus plants (up to 40 mM). Both asparaginase and asparagine aminotransferase activities were recovered in the 105,000 *g* (60 minute) supernatants. Low rates of asparagine transamination were also noted with soluble and peroxisomal tryptophan:glyoxylate/hydroxypyruvate aminotranferases, purified from spinach leaves (Noguchi and Hayashi 1980)

After the observation that asparaginase activity in many plants was potassium-dependant, other studies further explained the intermittent occurrence of asparagine metabolism in plants, noting that asparaginase and asparagine aminotransferase activities were localized spatially and temporally in the plant. For example, asparaginase activity was found to decrease with leaf age in *Pisum sativum*, from 100 nmol asp/min/g./fr./wt. in very young leaves to half this amount in half expanded leaves, and only trace levels were detected in mature leaves, while asparagine aminotransferase activity remained constant throughout leaf development (Ireland and Joy 1981). However, asparagine aminotransferase activity has been observed to increase in developing leaves of *Lupinus albus*, and decrease upon senescence (Atkins et al. 1983).

Ireland and Joy (1981) also reported that asparagine aminotransferase and asparaginase activities were present at low levels in pods, and that asparagine transaminase activity was absent in developing seed (in agreement with Sodek et al. 1980) or root extracts. Walton and Woolhouse (1986) have also reported activity of asparagine aminotransferase in *Pisum sativum* leaves, while cotyledon and root apices (apical 6 mm) exhibited trace activities. In a study on

asparaginase activity in two Lupin species, Chang and Farnden (1981) observed that with increasing distance from the tip of the root, asparaginase activity decreased rapidly (0 to 1 cm from the root tip asparaginase activity was 25 nmol/min/gm.fw.t, from 1 to 1.5 cm activity decreased by more than a half, from 1.5 to 2 cm no asparaginase activity was detected), and in young leaves asparaginase activity was high, while no activity was detected in mature leaves. The decrease of asparaginase activity with leaf age was also noted in studies with *Lupinus albus* (Atkins et al. 1983) and *Pisum sativum* (Ta and Joy 1985, Sieciechowicz et al. 1985). The absence of asparaginase in mature plant organs was also confirmed by Lea et al. (1984) using antisera prepared against asparaginase of developing *Lupinus polyphyllus* cotyledons. No asparaginase activity (Ouchterlony double immunodiffusion analysis) was detected in mature leaves, stems, roots or cotyledons of the same plant.

Support for asparagine metabolism in leaves with ^{15}N experiments

Feeding experiments corroborated the enzymological data, and confirmed that asparaginase and asparagine transaminase were the two main routes for asparagine metabolism in *Pisum sativum* leaves; as leaves developed, asparagine metabolism changed primarily from deamidation to transamination (Ireland and Joy 1981, Ta and Joy 1985, Ta et al. 1984a,b). For example, the fate of ^{15}N (amino)asparagine, fed via the transpiration stream at three stages of leaf development (young, half expanded and mature leaves), demonstrated that asparaginase activity decreased with leaf age because 1) the proportion of ^{15}N in aspartate, the deamidation product of asparagine, and glutamate, a transamination

product of aspartate, decreased with leaf age, 2) the addition of 5-diazo-4-oxo-L-norvaline (DONV, an inhibitor of several deamidases see Ta et al. 1984b) reduced ^{15}N recovery in aspartate and glutamate in young leaves more so than older leaves, and 3) addition of aminooxyacetate (AOA, an inhibitor of transaminases) had no effect on the recovery of ^{15}N in aspartate, yet reduced the presence of ^{15}N in glutamate in all leaf ages (see diagram below, Ta and Joy 1985). It was also noted that asparagine transamination may lead directly to homoserine synthesis since ^{15}N rapidly appeared in homoserine during feeding experiments and the incorporation of ^{15}N in homoserine was AOA sensitive (Ta et al. 1984a). This pathway of homoserine synthesis (i.e. transamination of 4-hydroxy-2-oxobutyric acid, Hoba, with asparagine, Pearson and Joy unpublished results) is different from the accepted route of synthesis, from aspartate, mediated by aspartate kinase, aspartate semialdehyde dehydrogenase, and homoserine dehydrogenase (Bryan 1980, Joy and Prabha 1986).

Other experiments feeding ^{15}N (amide)asparagine to half expanded leaves indicated that ^{15}N was recovered predominantly in hydroxysuccinamic acid (reduction product of oxosuccinamic acid, Lloyd and Joy 1978), and the amide group of glutamine. The recovery of ^{15}N in glutamine was greatly reduced by methionine sulfoximine (MSO, an inhibitor of glutamine synthetase activity, GS, see Mifflin and Lea 1982), while the levels of ^{15}N in NH_3 increased, consistent with deamidation and the inhibition of NH_3 assimilation by GS. When MSO and AOA were added together the recovery of ^{15}N in hydroxysuccinamic acid and NH_3 was reduced, i.e. deamidation occurred after the transamination of

asparagine (Ta et al 1984a,b)

These data suggested that transamination and deamidation of asparagine play an important role in supplying nitrogen to developing leaves (e.g. Ta et al. 1985). Since transamination of asparagine preceded deamidation (Ireland and Joy 1981, Ta and Joy 1985, Ta et al 1984 a), it was concluded that asparagine aminotransferase activity was more important than asparaginase activity in half expanded leaves, even though the *in vitro* activities of these enzymes were equivalent (Ireland and Joy 1981).

Asparagine and photorespiration

In more detailed studies on the enzymology of asparagine transamination in *Pisum sativum*, Ireland and Joy (1983a, b) suggested that the amino-N of asparagine could be incorporated into the photorespiratory pathway in developing leaves. This conclusion arose

from two observations. The first was that the asparagine aminotransferase responsible for metabolizing asparagine copurified with serine-glyoxylate aminotransferase, an important enzyme in the photorespiratory pathway (Tolbert 1980), after DEAE-Sephadex A25, Sephacryl S-300, and DEAE-sephacel chromatography (Ireland and Joy 1983a). The second observation was that asparagine aminotransferase activity co-sedimented and co-migrated along with catalase activity (a peroxisomal marker) during either differential centrifugation, or sucrose density gradient centrifugation (Ireland and Joy 1983b). This demonstrated that asparagine aminotransferase was located in the same subcellular compartment as the serine-glyoxylate aminotransferase. It was also concluded, in the latter study, that asparaginase was a cytosolic enzyme (Ireland and Joy 1983b).

The enzymological data, linking asparagine transamination with photorespiration, was supported by experiments feeding ^{15}N (amino)asparagine to developing leaves in conjunction with photorespiratory inhibitors (Ta and Joy 1985, 1986, Ta et al. 1985). After supplying ^{15}N (amino)asparagine through the transpirational stream, label was recovered in serine and glycine, intermediates of the photorespiratory pathway. When glycolate oxidase was inhibited with α -hydroxy-2-pyridine methanesulfonic acid (HPMS, thereby reducing the synthesis of glyoxylate), the recovery of ^{15}N in glycine decreased, while at the same time, label increased in alanine and glutamine. The inhibition of glycine decarboxylase and serine hydroxymethyltransferase, enzymes responsible for the conversion of glycine to serine, with isonicotinyl hydrazide (INH, a pyridoxal-P antagonist, Peterson 1982) led to an increase of ^{15}N in glycine (Ta et al. 1985). The increase or

decrease of ^{15}N in these and other amino acids was consistent with the scheme of photorespiration as it is currently thought to proceed (e.g. Tolbert 1980, Singh et al. 1985), and suggested that asparagine is involved in supplying nitrogen during photorespiration. The photorespiratory pathway was therefore proposed to be an open ended network of reactions supplying nitrogen for glycine, serine, and glutamate synthesis during the period when the leaf was still acquiring nitrogen for growth (Ta et al. 1985), rather than a closed cycle (e.g. Keys et al. 1978, Wallsgrove et al. 1983). Recent work has indicated that asparagine contributed approximately 7% of the nitrogen to glycine during photorespiration in half expanded leaves (Ta and Joy 1986).

The metabolic fate of oxosuccinamic acid and hydroxysuccinamic acid, which result from asparagine transamination, are not well understood in plants. Deamidation of oxosuccinamic acid in plants has been sporadically suggested in the literature (e.g. Meister 1953, Cinceroval 1969, Streeter 1977, Ta et al. 1984a,b) even though the enzyme α -amidase has not been isolated from plants (it has been characterized from rat liver, Hersh 1977). However, feeding experiments with ^{14}C -asparagine suggest that a strong equilibrium towards hydroxysuccinamic acid exists (Lloyd and Joy 1978). Deamidation of hydroxysuccinamic acid in crude extracts was only observed at low rates possibly indicating that hydroxysuccinamic acid may reconvert to oxosuccinamic acid prior to metabolism. However, the majority of the label from ^{15}N (amide)-hydroxysuccinamic acid, fed through the transpirational stream to half expanded pea leaves, was detected in the amide group of glutamine suggesting that deamidation of hydroxysuccinamic acid (observed to be DONV sensitive) and

reassimilation of NH_3 by glutamine synthetase (MSO sensitive) take place *in vivo*

Properties of plant asparaginases

To date there have been three reports on purified asparaginase from plant sources, two are from developing legume seeds (*Lupinus* species, Lea et al. 1978, Chang and Farnden 1981) and one is from a marine *Chlamydomonas* (Paul 1982). Several other reports on plant asparaginases have also listed some properties and these are presented below along with properties from several microbial asparaginases for comparison (*Leptosphaeria michotii*, *E. coli* (EC-2) and *Saccharomyces cerevisiae* I and II).

Source	M _r	K _m (mM)	pH opt.	reference
<i>L. polyphyllus</i> ^a	72,000	12.2	broad	Lea et al. 1978
<i>L. arboreus</i> ^a	75,000	6.6	-	Chang and Farnden 1981
<i>L. angustifolius</i> ^a	75,000	7.0	-	"
<i>P. sativum</i> ^a	68,300	3.2	-	Sodek et al. 1980
" leaf	-	8.0	-	Ireland and Joy 1981
" seed	-	3.0	-	"
<i>A. officinalis</i>	125,000	-	8.5	Cooney et al. 1980
<i>Chlamydomonas</i>	275,000	0.13	broad	Paul 1982
<i>L. michotii</i>	140,000	-	broad	Jerebzooff-Quintin and Jerebzooff 1985
<i>E. coli</i>	130,000	0.13	broad	Wriston 1985
<i>S. cerevisiae</i> I	400,000	0.74	8.5	Dunlop et al. 1978
" II	800,000	0.35	6.8	"

a) enzyme obtained from developing seeds approximately 3 weeks after anthesis

Asparaginase appears to be a cytosolic enzyme and is detected in the soluble fraction after centrifugation from all higher plant sources examined so far. Cooney et al. (1981) reported that asparaginase

activity from seedlings was recovered in the supernatant fraction after a 105000 *g*, 60 minute centrifugation period Ireland and Joy (1984b) also determined that asparaginase was soluble (recovered in the supernatant after 20000 *g*, 10 minute centrifugation), as did Lea et al (1978) following unsuccessful attempts to determine the subcellular localization of asparaginase using sucrose density gradient centrifugation. In several microorganisms, for example in yeast or *E. coli*, two asparaginases are present, an internal constitutive enzyme and an external isoform (Arima et al 1972). In *Chlamydomonas* only an extracellular asparaginase is present and is thought to reside in the periplasmic space (Paul and Cooksey 1979, Paul 1982).

Asparaginase obtained from developing Lupin seeds was inhibited from 90 to 100% by salts of transition elements such as cobaltic, ferric, nickel, cadmium, and cupric (8.3 mM, supplied as Cl^- salts), while compounds such as ATP, ADP, AMP, NADH, and NADPH had no effect on activity (Lea et al. 1978). Glycine inhibited asparaginase (K_i 5.2 mM) from developing lupin seeds, however the physiological levels of glycine in this tissue were approximately 3.0 mM (Chang and Farnden 1981). Two other powerful inhibitors (i.e. more than 90% inhibition) of asparaginase activity are 3-cyano-alanine (25 to 30 mM, Lea et al. 1978, Chang and Farnden 1981) and DONV (20 to 30 mM, Lea et al. 1978, Ta et al. 1984b).

Microbial asparaginase and its regulation

The occurrence of extracellular asparaginases in microorganisms, in addition to internal asparaginases, is well documented (Arima et al. 1972). Studies by Dunlop, Roon and co-workers demonstrated that several strains of *Saccharomyces cerevisiae* possessed an external

asparaginase, termed asparaginase II, that increased in activity during nitrogen starvation (Dunlop and Roon 1975, Dunlop et al. 1976). The increase in asparaginase II activity required the presence of carbohydrate (glucose, fructose, or mannose), and was inhibited by 1 μ g/ml cycloheximide. Therefore, the authors suggested that asparaginase II synthesis was derepressed during nitrogen starvation (Dunlop and Roon 1975, 1980). Nitrogen catabolite repression (NCR) regulates the activity of a number of yeast enzymes (Dubois et al. 1974, 1977, Roon et al 1982). Several hypotheses on the mechanism of NCR have been proposed, although to date none have been supported (Roon et al. 1982). For example, it was suggested that direct interaction of NADP-glutamate dehydrogenase (NADP-GDH), as a repressor molecule, with a regulatory gene could account for NCR, since mutants lacking NADP-GDH (*gdh A* mutation) were insensitive to NH_3 repression of NCR regulated enzymes (arginase, allantoinase, ornithine transaminase, urea amidohydrolase, and asparaginase II, Dubois et al 1974, Dunlop et al. 1980). However, the addition of amino acids to yeast with the *gdh A* mutation resulted in the repression of asparaginase II synthesis, suggesting that the *gdh A* mutation may affect the system through a metabolic block rather than in a regulatory manner (Dunlop et al 1980). Further work indicated that the growth state of yeast along with nitrogen nutritional status affected asparaginase II synthesis by NCR through amino acids rather than NH_3 , although the mechanism is still not understood (Roon et al. 1982).

Asparaginase I, a constitutive (internal) enzyme in yeast, was not affected by nitrogen starvation, however it displayed a sigmoidal

response to increasing levels of L-asparagine, asparaginase II responded in a hyperbolic manner (Dunlop et al. 1978). It was proposed that asparaginase I is only active when asparagine concentrations are high so that asparagine pools, required for protein synthesis, are not depleted during active growth (Dunlop et al. 1978).

Paul and Cooksey (1979, 1981a) reported that a marine *Chlamydomonas* species could use asparagine or glutamine as sole nitrogen sources (in addition to inorganic nitrogen sources). Under conditions of limiting nitrogen (NO_3^- , NH_3 or asparagine) intraperiplasmic (possibly external) asparaginase, and constitutive glutamine synthetase, were observed to increase in activity, while NADH- and NADPH-glutamate dehydrogenase activities decreased (Paul and Cooksey 1981a, b). They concluded that *de novo* protein synthesis was responsible for the increase in asparaginase activity (approximately 4-fold during a 48 hour period). The increase in asparaginase activity was dependant upon the energy fluence rate, and inhibited by 2 μM DCMU, or 10 $\mu\text{g}/\text{ml}$ cycloheximide. They suggested that the mechanism regulating *Chlamydomonas* asparaginase paralleled the mechanism functioning in yeast (Paul and Cooksey 1981a). Nitrogen catabolite repression also appears to regulate asparaginase activity in *Aspergillus nidulans* (Drains et al. 1977) and *Klebsiella aerogenes* (Resnick and Magasanik 1976).

Studies examining sporulation rhythms in *Leptosphaeria michotii* indicated that regulation of these rhythms occurred at two levels. The first level of regulation involved translation on 80S ribosomal proteins, since period shifts in protein synthesis and enzyme activity were sensitive to cycloheximide and puromycin. The second level of regulation

regulation involved the "asparagine-aspartate-oxaloacetate-pyruvate pathway" since three enzymes (asparaginase, aspartate transaminase and alanine transaminase) oscillated with periods identical to that of the sporulation rhythm, and modification of their activities affected the sporulation cycle (Jerebzoﬀ and Jerebzoﬀ-Quintin 1983a,b). Addition of cycloheximide at low concentrations (10 μ M) altered the phase of the sporulation cycle, and reduced the amplitude and phase of aspartate and alanine transaminase activity but low cycloheximide concentrations had no effect on asparaginase activity. Asparaginase activity was modified at high (50 μ M) cycloheximide concentrations (oscillations in asparaginase activity persisted, only the period shortened), suggesting that protein synthesis partially regulated enzyme activity.

Further analysis demonstrated that two forms of asparaginase (as separated on DEAE-Sephacel) were present in *Leptosphaeria michotii*. An active asparaginase (termed form I) oscillated with the same period and phase as total asparaginase activity, while asparaginase-form 2 (which exhibited only 25% of form I activity) varied with a similar period, but was out of phase by 180° (Jerebzoﬀ-Quintin and Jerebzoﬀ 1985). Both forms displayed sigmoidal kinetics, however the half maximal velocity ($S_{0.5}$) of form I varied inversely with asparaginase activity. These authors suggested that an inhibitor or activator may bind asparaginase-form I during extraction, modifying *in vitro* activity, since experiments indicated that post-translational modification of asparaginase-form I through association with a protein-complex (and possibly reversible phosphorylation, unpublished data) may take place (Jerebzoﬀ-Quintin and Jerebzoﬀ 1985).

In microbial systems, regulation of asparaginase activity appears to

involve *de novo* synthesis of the enzyme. Under conditions of low external nitrogen, the synthesis of asparaginase is derepressed, and in a *Chlamydomonas* sp. asparaginase synthesis has been shown to be dependant on non-cyclic photophosphorylation.

Regulation of enzymes by light

During the course of the present investigation on asparaginase activity in leaves of *Pisum sativum* it was observed that asparaginase activity varied diurnally (increasing in light, see results section I), and indicated that asparaginase activity was regulated in some manner

Several other enzymes of nitrogen metabolism in plants have been found to vary during the photoperiod, however some differences are noted between species. For example, glutamate dehydrogenase (GDH) and nitrate reductase (NR) oscillated (out of phase with each other) with an ultradian (less than 24 hour) period in *Glycine max* and *Zea mays* leaves (GDH was more active in the dark than in the light, Duke et al. 1978), glutamine synthetase (GS) activity oscillated (and was correlated with oscillations of energy charge) with an ultradian period in *Helianthus annuus* roots (Knight and Weissman 1982), NR, glutamate synthase (GOGAT), and aspartate transaminase activities varied diurnally in *Hordeum vulgare* leaves (GS and alanine transaminase activities remained essentially constant, Lillo 1984a), and arginine decarboxylase in *Chenopodium rubrum* varied diurnally (Schwall et al. 1985). In all of cases cited above, a circadian rhythm of enzyme activity was displayed because the rhythms persisted during a regime of continuous light (and in some cases continuous dark).

Two general hypotheses, variants on a similar theme, have been put

forth to explain the oscillatory nature of rhythmic phenomena in plants, and in eukaryote organisms in general (since prokaryotes show no circadian rhythms, Wagner 1977). Implicit in both hypotheses is the idea that a mechanism somehow alters the properties or structure of the membrane (Heide 1977, Wagner 1977). One hypothesis, which is applicable to eukaryotic organisms, maintains that changes in the adenylate energy charge (AEC, an index of the readily available energy; defined as the ratio of anhydride phosphate bonds of the adenylate pool compared to the total adenine nucleotide concentration, Pradet and Raymond 1983) alters membrane structure (Wagner 1977), and the other hypothesis, applicable to plants only, involves an interaction of phytochrome with the membrane (Heide 1977).

Regulatory functions of biomembranes (including associated proteins, ATPases, translocases etc.) can affect cellular processes in several ways: a) development of an electrochemical gradient leads to changes in the adenylate energy charge (AEC) of the cell or organelle, b) compartmentation of metabolic processes allows reactions to take place in opposite directions without futile cycling, and also controls metabolite translocation (e.g. Heber 1974), and c) changes in membrane permeability, for example by phytochrome (Heide 1977, McCurdy and Pratt 1986), would allow increased Ca^{2+} concentration in the cell (Roux et al. 1986).

Light-dependant increases of enzyme activity have been reported to operate through many different mechanisms, all of which are ultimately a result of a membrane-dependant process. Several mechanisms of enzyme regulation are outlined below and can be grouped into four broad categories: 1) regulation via low molecular weight compounds; for

example: adenylates, pyridine nucleotides, ions, and substrates or products, 2) protein-mediated regulation for example: thioredoxin, light effect mediator, kinase, and calmodulin, 3) *de novo* protein synthesis, and 4) protein degradation. In most cases more than one mechanism accounts for the increase (or decrease) in enzyme activity.

Several enzymes localized in the chloroplast increase in activity in the light, for example NADP-glyceraldehyde 3-phosphate dehydrogenase (NADP-GAPD), ribulose biphosphate carboxylase oxygenase (Rubisco), ribulose 5-phosphate kinase (PRK), 3-phosphoglycerate kinase (PGK) (Anderson 1979, Buchanan 1980), and GS (chloroplast form, GS₂, O'Neal and Joy 1975, Stewart et al. 1980), are controlled by low molecular weight compounds, in part due to control through AEC (Pradet and Raymond 1983). For example, Zeigler and co-workers reported that incubation of NADP-GAPD with ATP and NADPH (in the presence of Pi) activated the enzyme slowly (Buchanan 1980). Several metabolites including ATP, NADPH, and NADH are reported to increase in concentration upon illumination, however the extent of change of these metabolites is organelle-specific (for example, upon illumination chloroplast and cytosolic ATP levels increase rapidly; while mitochondrial ATP levels decrease, Hampp et al. 1985, Stitt et al. 1985). The possibility that changes in adenylate levels may be regulated in some manner by phytochrome has been suggested, and questioned, in the literature (Quail 1983).

Many enzymes involved in nitrogen metabolism are regulated (through inhibition and activation) by end products, for example aspartate kinase, threonine synthase, threonine dehydratase, and homoserine dehydrogenase, to name a few (Bryan 1980). This mechanism is one that

involves mediation of activity through low molecular weight compounds, however regulation of enzyme activity by low molecular weight compounds as a direct result of a light-dependant mechanism is not well documented. Synthesis of the aspartic family of amino acids is stimulated in the light, implying that light-dependant regulatory systems may operate (Mills et al. 1980). This also appears to apply for enzymes involved in nitrogen assimilation. Glutamine synthetase, GOGAT and nitrite reductase (NiR) are located in the chloroplast, and are directly dependant upon products of photosynthesis for activity (ferredoxin, ATP, and NADPH). The reactions catalyzed by these enzymes have been considered 'photosynthetic reactions' (Wallsgrave et al. 1983).

Nitrate reductase has also been reported to be regulated by the AEC because a high ATP/ADP ratio would 'switch off' the mitochondrial sink for NADH, needed for NR activity (Sawhney et al. 1978, Naik and Nicholas 1986). However, this suggestion has been criticised by Reed and Canvin (1982) who demonstrated that increased supply of NAD(P)H in the light would satisfy both NR and mitochondrial respiration, if in fact mitochondrial respiration occurs in the light (Canvin et al. 1980, Abrol et al. 1983, Singh et al. 1985). Other compounds have also been implicated in the regulation of NR activity. For example Solomonson and Spehar (1977) have suggested that cyanide may inhibit NR activity in *Chlorella*, an observation supported in higher plants as well (Huckelsby et al. 1982). The involvement of cyanide and NADH in the regulation of NR activity has also been proposed (Pistorius et al. 1976, Maldonado et al. 1982). Phenylalanine ammonia-lyase activity has also been reported to be modulated by low molecular weight compounds, including end product inhibition (in addition to a variety of other mechanisms, Camm

and Towers 1973, Hanson and Havir 1981)

Photosynthetic CO_2 fixation is regulated by changes in stromal pH (Werdan et al. 1975, Heldt 1979). The change in pH between the stroma and interthylakoid space (estimated to reach up to 2.5 units), results from light-dependant proton transport from the stroma across the thylakoid membrane into the thylakoid space (Heldt et al. 1973). A result of proton transport across the thylakoid membrane is that the stromal Mg^{2+} concentration increases approximately 3-fold (Portis et al. 1976, Heldt 1979). Several enzymes of the reductive pentose phosphate pathway, fructose 1,6-bisphosphatase (FBPase), sedoheptulose 1,7-bisphosphatase (SBPase) (Flugge et al. 1980), Rubisco (Akazawa 1979), and nitrogen metabolism, GS_2 (O'Neal and Joy 1975, Lea and Mifflin 1979, McNally and Hirel 1983) are reported to increase activity under conditions of increased alkalinity and Mg^{2+} concentrations. However the importance of the increase in stromal Mg^{2+} , with respect to the activities of these enzymes of the reductive pentose phosphate pathway, has been questioned (Buchanan 1980, Pradet and Raymond 1983).

Another mechanism controlling several chloroplast and cytosol enzymes involves the interaction with protein mediators. Protein mediators affect enzyme activity through covalent modification by reducing disulfide (S-S) groups, or phosphorylating serine, threonine or histidine (in the case of pyruvate di kinase) residues. Reversible reduction of thiol groups will be considered first.

Two mechanisms have been reported to reversibly modify enzyme activity through a mechanism of reduction-oxidation of disulfide bonds. One mechanism, proposed by Buchanan and co-workers, involves two

soluble proteins, ferredoxin-thioredoxin reductase, and thioredoxin (Buchanan 1980, Jacquot 1984). The second mechanism regulating enzyme activity through S-S reduction was suggested by Anderson and co-workers and also involves two factors: a membrane bound light effect mediator (LEM), and a soluble protein modulator (essentially a reductase; Mr 51000, Anderson et al. 1980, Jacquot 1984). In an effort to reconcile differences between the ferredoxin-thioredoxin reductase and LEM mechanisms of enzyme regulation, several studies have demonstrated that thioredoxins and to some extent ferredoxin are loosely associated with photosynthetic membranes (e.g. Scheibe and Beck 1979), and methods of preparation may have led to the discrepancies noted. It is possible that the differences are academic and Jacquot (1984) suggested that LEM be considered a 'pseudo ferredoxin-thioredoxin reductase'.

The reducing side of photosystem I has been implicated in supplying electrons for reduction of LEM, because disulfide reduction was DCMU (an inhibitor of photosynthetic electron flow between photosystem II and the plastoquinone pool, Trebst 1980) sensitive (Anderson et al. 1980). Two LEM's are considered to be involved in S-S reduction of enzymes, LEM_I accepts electrons from photosystem I before ferredoxin, while LEM_{II} accepts electrons at the level of ferredoxin (Anderson 1979). Two thioredoxins (both of which accept electrons at the level of ferredoxin) have been detected in the chloroplast, thioredoxin_r (regulating FBPase), and thioredoxin_m (regulating NADP-malate dehydrogenase, NADP-MDH), which has also been detected in the cytosol (Jacquot 1984). Several enzymes have been reported to be modified by thioredoxin/LEM: FBPase, SBPase, NADP-MDH, NADP-GAPD, PRK, G6PD, (Anderson 1979, Buchanan

1980, Jacquot 1984), phenylalanine ammonia-lyase (PAL, chloroplast form, Nishizawa et al. 1979), GS (Cullimore 1981, Stewart et al. 1980), GS and GOGAT (Tishner and Schmidt 1982). Reduction of disulfide bonds *in vitro* can be achieved with the addition of the sulfhydryl compound dithiothreitol (DTT, Buchanan 1980). The exact mechanism accounting for the oxidation of the thiol groups is still under debate. Glutathione (oxidized form: GSSG, reduced form GSH) has been suggested as a possible candidate for oxidation of sulfhydryl groups on enzymes (Wolosiuk and Buchanan 1977), however Halliwell and Foyer observed that the GSH/GSSG ratio remained high (10/1) both in the light and in the dark (see Jacquot 1984).

All of the above enzymes, except G6PD, increased in activity as a result of disulfide reduction in the light within minutes (from 1 to 30 minutes with purified enzyme preparations in the presence of DTT, or illuminated isolated chloroplasts, Anderson and Avron 1976, Scheibe and Anderson 1981, Leegood and Walker 1982). Glucose 6-phosphate dehydrogenase, the initial enzyme in the oxidative pentose phosphate pathway, was inactivated in the light within minutes in the presence of LEM (Anderson 1979, Scheibe and Anderson 1981) or reduced ferredoxin (Buchanan 1980). In addition to thiol regulation of activity, Anderson has also demonstrated that disassociation of G6PD with chloroplast membrane fractions reduced activity (Ben-Bassat and Anderson 1981). A similar mechanism (i.e. partitioning of the enzyme between the soluble and particulate phase) may also play a role in modulating NAD(P)-GAPD (Anderson et al. 1980), NADP-MDH (Scheibe and Beck 1979), and PRK (Rischer and Latzko 1979, Anderson et al. 1980) activities.

Reversible phosphorylation of proteins involves the action of two

Mg^{2+} dependant enzymes, a protein kinase and a phosphoprotein phosphatase. Nuclear proteins (both histone and non-histone) are extensively phosphorylated, and phosphorylation of non-histone and histone proteins have been implicated in the regulation of gene activity and cell division (Trewavas 1976). Several plant enzymes are reported to be regulated through reversible phosphorylation. For example, the pyruvate dehydrogenase complex (PDC) was inactivated (in minutes) in the presence of 1 mM ATP and reactivated with 10 to 20 mM Mg^{2+} . The reactivation of PDC was inhibited in the presence of NaF (3mM, an inhibitor of phosphoprotein phosphatases), and it was therefore concluded that PDC was inactivated through the covalent bonding of a phosphoryl group (Rao and Randall 1980). Studies with quinate:NAD⁺ oxidoreductase (QNOR) demonstrated that the enzyme was stimulated upon phosphorylation. Activity was reduced upon incubation with Mg^{2+} (10 mM, i.e. enhancing phosphatase activity), and was prevented by NaF (50 mM). Once inactivated, the addition of MgATP (4mM) increased activity. Purified QNOR incubated with protein kinase, and [γ - ^{32}P]ATP increased activity and became labelled with $^{32}PO_4^{3-}$ (Refeno et al. 1982).

Pyruvate, Pi dikinase (PDK) has also been reported to be reversibly phosphorylated on two residues (threonine and histidine) in a rather complex process controlled by 'PDK regulatory protein' (Edwards et al. 1985). Another well characterized system in plants known to respond to reversible phosphorylation involves the light-harvesting chlorophyll *a/b* complex (Bennett 1983). In yeast, NAD-GDH is subject to reversible phosphorylation. The phosphorylated enzyme displayed reduced activity (K_m increased from 20 to 128 mM) and a lower pH optimum (decreased

from 8.2 to 6.2, Hemmings 1982)

In *E. coli*, GS is regulated by means of reversible adenylation by an adenylyltransferase (ATase), in a process regulated by the levels of ATP, oxoglutarate, and glutamine by means of the interaction of the ATase and a small regulatory protein. High levels of glutamine promote adenylation, reducing GS activity, while high levels of oxoglutarate or ATP stimulate deadenylation, increasing GS activity. The small regulatory protein itself is subject to regulation via uridylation, when uridylated deadenylation is enhanced and when deuridylated, adenylation is favoured. However, uridylation is controlled by a uridylyltransferase, the activity of which is modified by the levels of oxoglutarate and glutamine (Stewart et al. 1980). This system of GS regulation has not been found in plants (Kingdon et al. 1967).

Calmodulin is a heat-stable Ca^{2+} binding protein that has been detected in many plants and reported to activate, when bound to Ca^{2+} , NAD kinase, Ca^{2+} -dependant ATPase, QNOR (Marme 1985), and aspartate kinase (Sane et al. 1984). The involvement of Ca^{2+} in the regulation of protein kinase activity (Blowers et al. 1985), and Ca^{2+} -calmodulin control of protein phosphorylation (Veluthambi and Poovaiah 1984) has led to a proposal of a two-step enzyme cascade system in plants. For example, in crude preparations QNOR phosphorylation was observed in the presence of MgATP (as mentioned above), however if calmodulin-free QNOR was incubated with MgATP, no increase in activity was noted unless Ca^{2+} -calmodulin was added to the preparation. The two step cascade system proposed was that Ca^{2+} bound calmodulin, the active Ca^{2+} -calmodulin complex interacted with protein kinase and the active kinase, in the presence of ATP, phosphorylated QNOR thereby stimulating

activity (Ranjewa et al. 1984). No Ca^{2+} -calmodulin dependant phosphoprotein phosphatases have been detected in plants (Roux et al. 1986)

Calcium-calmodulin dependant Ca^{2+} ATPase activity, which functions in transporting Ca^{2+} out of the cytosol, is under phytochrome control. Calmodulin regulation of the Ca^{2+} -ATPase is reduced in light (over a period of approximately 30 minutes in isolated microsomal fractions, Marme 1985), while at the same time, light (through a phytochrome regulated process) has also been implicated in promoting Ca^{2+} influx within minutes (Roux et al. 1986). Upon illumination, increased Ca^{2+} levels in the cytosol and chloroplast would lead to direct or indirect effects on enzyme activity through interactions with calmodulin. An example of a direct effect on enzyme activity by Ca^{2+} -calmodulin is enhanced kinase activity, as in the case for QNOR; an indirect effect would arise through stimulated NAD kinase activity which would alter the pyridine nucleotide levels (NAD^+ and NADP^+) in the cytosol and chloroplast. Indirect modulation of enzyme activity through Ca^{2+} -calmodulin stimulated NAD kinase has been suggested for the regulation of NAD(P)-GAPD (Goto 1984).

Light-dependant increase of enzyme activity may also be a result of light induced *de novo* protein synthesis. Exposure of etiolated seedlings to light results in a transformation of etioplasts to chloroplasts, along with dramatic increases in protein synthesis. Increased enzyme activity during greening has been demonstrated for phosphoenolpyruvate carboxylase, Rubisco (Lamb and Lawton 1983), PAL (Hanson and Havir 1982), NR (Duke and Duke 1984), NiR (Gupta and Beevers 1985), GS_1 (cytosolic GS, Harel et al. 1982), GS_2 (plastid GS,

Tobin et al. 1985) and GOGAT (ferredoxin dependant isoform, Wallsgrove et al. 1982, Suzuki and Gadal 1984). It has been well established that exposure of etiolated seedlings to light increases the proportion of ribosomes present as polysomes over a period of 2 to 10 hours (Smith 1976, Mosinger and Schopfer 1983, Lamb and Lawton 1983). These experiments are possibly an indication of increased mRNA synthesis, however increased mRNA levels have only been demonstrated recently (Tobin and Silverthorne 1985). In many cases the increase of mRNA levels (and polysome levels) is regulated through phytochrome (e.g. Mosinger and Schopfer 1983), although the exact nature of the mechanism is not understood (Lamb and Lawton 1983, Harpster and Apel 1985). It is currently thought that increases in mRNA levels are due to increased rates of transcription and not activation of preexisting mRNA (Harpster and Apel 1985). Recently Kloppstech (1985) demonstrated that the level of three mRNA's (coding for the light-harvesting chlorophyll *a/b* protein, LHCP, the small subunit of Rubisco, ssRubisco, and an unknown protein) varied in a circadian manner and were not regulated by phytochrome. This is an interesting observation since increased levels of mRNA for ssRubisco and LHCP in greening tissues are usually considered to be under phytochrome control (Tobin and Silverthorne 1985), and therefore Kloppstech (1985) suggested that the greening situation may be an exception rather than the rule.

Protein degradation through proteolytic activity leads to irreversible inactivation of enzymes (Wallace 1977). Several enzymes involved in plant nitrogen metabolism have been reported to be susceptible to inactivation in crude extracts, for example NR, GS, and GOGAT (GDH was apparently more stable, Streit and Feller 1983). The factor(s) reducing

activity were precipitated with ammonium sulfate, heat-labile and excluded by Sephadex G-25, while enzyme inactivation was delayed with the addition of casein or with the addition of a heat-inactivated preparation (Streit and Feller 1983). Nitrate reductase from several plants was also found to be susceptible to proteolysis ('inactivating enzymes'), however relief from proteolysis by adding phenylmethylsulphonyl fluoride (PMSF, an inhibitor of proteases) was observed in only a few cases (Wallace 1977). In maize an inhibitor of NR inactivating enzyme has also been suggested (Wallace 1977). Somers et al. (1983) also concluded that the decrease in NR activity was due to proteolysis and not reversible inactivation. A diurnal variation of NR stability in crude extracts obtained from wheat leaves has also been reported, and three factors affecting NR activity detected (Sherrard et al. 1979). A diurnal variation in the three factors was noted, however only one factor (an inhibitor of NR activity) increased activity that coincided with decreased NR activity (Sherrard et al. 1979).

Limited proteolysis of an enzyme may also lead to modification of activity, as reported for plant glucan synthase where activity increased in minutes in the presence of an endogenous protease or added trypsin (Kauss et al. 1983), and yeast NAD-GDH (incubation of phosphorylated enzyme with trypsin increased activity, Hemmings 1982). The mechanism of limited proteolysis has been examined in some detail in animal systems (Cohen 1983). Limited proteolysis and stimulation of activity may also include processing of newly synthesized proteins by excision of leader sequences (as in the signal hypothesis of polypeptide transport across membranes) during 'enzyme maturation' (Lamb and Lawton 1983).

Aggregation and dissaggregation of subunits may affect enzyme activity. A decrease in M_r of NADP-GAPD in the presence of ATP and NADPH accompanies an increase in activity (Buchanan 1979, Latzko and Kelly 1979). However the change in M_r may be artifactual because de Looze and Wagner (1983) have observed that NADP-GAPD aggregates with ribonucleoproteins in a pyridine dependant manner (NADP⁺ promotes dissaggregation, NAD⁺ enhances aggregation) during extraction. With increasing pH, FBPase undergoes a decrease in M_r , from tetramer (M_r 140,000) to dimer (M_r 70,000), and the FBPase dimer may attain SBPase activity (Latzko and Kelly 1979). Asparagine synthetase obtained from lupin cotyledons also undergoes a change in M_r *in vitro*, increasing M_r (from 160,000 to 320,000) in presence of ATP and Mg^{2+} (Rognes 1975).

In summary, asparagine is a nitrogen transport compound found in many plants. It is synthesized by asparagine synthetase upon germination, when protein reserves are hydrolyzed in cotyledons, or in roots during routine nitrogen assimilation. Asparagine synthetase has not been isolated from other plant organs, however evidence suggests that asparagine is synthesized in leaves. Asparagine is metabolized by two separate pathways, deamidation and transamination releasing nitrogen for amino acid and protein synthesis. Asparaginase activity in many plant species is potassium dependant and is active for short periods of time in regions requiring nitrogen for growth such as developing seeds, roots, and leaves. Asparagine aminotransferase activity is present in developing and mature leaves, possibly supplying nitrogen for leaf growth through the photorespiratory pathway, and is not found in other organs. In older leaves asparagine is not required

there for growth, and several reports have suggested that transpirationally derived asparagine in older leaves undergoes little metabolism and is reexported to the apex, or developing fruits.

Asparaginase activity undergoes diurnal variation in activity in developing leaves, and modification of asparaginase activity has also been observed in microorganisms. In microorganisms protein synthesis appears to play an important role in the increase of asparaginase activity, however phosphorylation has also been suggested.

This study examines the occurrence and regulation of asparaginase activity in developing pea leaves.

Materials and Methods

Pisum sativum L. (c.v. Little Marvel, No. 1, McKenzie Steele Briggs, Brandon Manitoba Canada, R7A 6E1), were grown either hydroponically or in a soil mixture. Seeds were surface sterilized in 5% (v/v) sodium hypochlorite for 10 minutes, rinsed with several washes of tap water until no odour from the hypochlorite remained, and soaked in aerated tap water for 24 hours. The following day the seeds were placed into water-soaked vermiculite and left in the dark. After 5 days, germinated seedlings (roots 8-10 cm. long), were placed into aerated, 1/2 strength Hoaglands nutrient solution (see appendix 1) which was replaced at weekly intervals. Cotyledons were removed from hydroponically grown plants when they had three fully expanded leaves (14 days after imbibition). For soil grown plants, sterilized and soaked seeds (see above) were planted directly into a soil-perlite mixture (1 part perlite, 1 part peatmoss, and 1 part sand by volume) and transferred to growth cabinets. The soil grown plants were watered with tap water every second day and no other source of nutrient was supplied; cotyledons were not removed from soil grown plants.

Plants grown hydroponically did not become nodulated, while soil grown plants were observed to occasionally be nodulated. Both soil and hydroponic plants were grown in growth cabinets with a 16 hour light period at 23°C, and 8 hours of dark at 18°C. The light source used in the growth cabinets was a combination of both incandescent (60 watt, Westinghouse) and fluorescent (Phillips F 96 T 12/CW/HO or

Westinghouse Cool White: SHO F48 T12/CW/HO). The energy fluence rate, measured as photosynthetically active radiation, was 10 Watts/M².

Chemicals

Chemicals used were reagent grade from either Sigma, Baker, Fisher, or BDH Chemical Companies. Amino acids were from Sigma Chemical Co., ¹⁴C-leu (specific activity: 42 mCi/mmol, 1.55 GBq/mmol) was obtained from Amersham. Dowex 1 x8 200-400, Cl⁻ form (capacity: 3.2 meq/dry gm), and DEAE Cellulose (Cellex D, high capacity) were purchased from BioRad. Sephadex G-25 (50-150μ fine or medium and PD-10 columns) and Sephacryl S300 were bought from Pharmacia Fine Chemicals. Glutamate dehydrogenase was obtained from Boehringer Mannheim. Asparagine, homoserine, and glutamine were added to amino acid calibration standards for both the Beckman amino acid analyser used on physiological mode (Hamilton 6, type P-AN acid neutrals standard), and the Waters HPLC, or the Beckman on hydrolysate mode (Sigma amino acid standard).

Methods

1) Enzyme extractions:

All procedures were carried out at ~4°C, and unless otherwise noted, developing fifth leaves (counted from the base of the plant; plants 15-18 days old) were used as enzyme source. Leaf material was weighed, and ground in 3 - 5 volumes of appropriate extraction buffer in a mortar and

pestle for about 30 seconds until a uniform macerate was obtained. This brei was passed through 4 layers of moistened cheese cloth and centrifuged for 10 minutes at 20,000 *g*. The volume of the supernatant was recorded and either 1 ml of the supernatant passed through Sephadex G-25 (PD-10, equilibrated in appropriate final buffer); or the entire supernatant was brought to 65% saturation with $(\text{NH}_4)_2\text{SO}_4$ (previously ground to a fine powder) and stirred vigorously for 10 minutes (4°C). The $(\text{NH}_4)_2\text{SO}_4$ precipitate was recovered by a second 20,000 *g* spin for 10 minutes and resuspended in 1 volume (original fresh weight) of the extraction buffer. Protein concentrations were estimated by the method of Bradford (1976). Average time taken, from enzyme extraction to initiation of assay, was 30-45 minutes. Enzyme activity is expressed as amount of product produced/min./gm fresh weight or mg protein.

Asparaginase

Leaf material was ground in either 50 mM Tris HCl (pH 8.0) containing 50 mM KCl $\pm$ 2 mM DTT or 50 mM potassium phosphate (pH 8.0) containing 10% glycerol $\pm$ 2 mM DTT. Asparagine (100 mM stock solution) was passed through Dowex 1 (acetate form pH 6.8, Prusiner and Milner 1970) to remove any contaminating aspartate (Lea and Mifflin 1975). Assay mixtures contained non-saturating concentrations of asparagine (20 mM, pH 8.0), extraction buffer, and enzyme (approximately 3 mg protein, or 60-70 mg fresh weight) in a total volume of 0.25 ml. Mixtures were incubated at 34°C for 30 minutes. Controls, containing assay mixtures

without enzyme or lacking substrate, were also run. Reaction mixtures were assayed for either aspartate or NH_3 at the beginning (0 time) and after 30 minutes incubation. For the analysis of aspartate, 25 mg of 5-sulfosalicylic acid (in a volume of 0.5 ml) was added to precipitate proteins (12,000 g for 3 minutes) and 0.2 ml of the supernatant removed and analyzed either by HPLC, or amino acid analysis (with lithium-citrate buffers; physiological mode). If NH_3 was to be determined, 0.2 ml aliquots of the reaction mixture were removed for microdiffusion (see below). The production of NH_3 was stoichiometric with aspartate synthesis in assay mixtures, as determined by microdiffusion or amino acid analysis respectively. Assays were linear for well over the 30 minute assay period, and over a range of fresh weight/protein concentrations (0.01-0.1 gm fr. wt. or 1-4 mg protein/0.25 ml assay solution; highest assayed).

Asparagine aminotransferase

The method used was that of Ireland and Joy (1981). The initial 20000 g supernatant (plant material extracted in 50 mM Tris HCl, pH 8.0, and 2 mM DTT) was brought to 18% PEG (8000, by addition of a 50% (w/v) solution of PEG in buffer), and centrifuged at 20000 g for 10 minutes. The pellet was resuspended in homogenization buffer. The assay mixtures contained 20 mM asparagine, 20 mM pyruvate, 2.7 μM pyridoxal 5'-phosphate, 50 mM Tris HCl, and enzyme in a total volume of 0.3 ml (at 34°C). At 0 and 30 minutes, reactions were terminated with the addition of 25 mg of 5-sulfosalicylic acid, centrifuged at 12,000 g

for 3 minutes and the amount of alanine produced determined by amino acid analysis.

NADH and NAD⁺ Glutamate dehydrogenase

The methods used were adapted from those of Joy (1974) and Nauen and Hartmann (1980). Leaf tissues were extracted and desalted in 50 mM Tris HCl, pH 7.8, 10 mM MgSO₄, 1 mM EDTA, and 1 mM DTT. For assaying reductive amination of oxoglutarate, 0.1 ml aliquots of the extract were added to mixtures to give final concentrations of 50 mM Tris HCl, 1 mM CaCl₂, 13 mM oxoglutarate, and 60 mM (NH₄)₂SO₄. The reactions were started by the addition of 0.3 mM NADH (total assay volume 1.2 ml), and the decreases in A₃₄₀ were followed over a 5 minute period at 25°C. Oxidative deamination of glutamate (NAD⁺-GDH) was assayed in solutions containing 75 mM Tris HCl, pH 8.0, with 33 mM glutamate (final concentrations), and the reaction started by adding 2.1 mM NAD⁺. The increase in A₃₄₀ was followed for a 10 minute period at 25°C. Total volumes of assays were 1.2 ml. Controls, without enzyme, or substrates, were also run with both enzyme assays.

Glutamine synthetase (biosynthetic assay)

The method used for the analysis of glutamine synthetase was that of O'Neal and Joy (1973). Leaf material was ground in 50 mM Tris HCl (pH 8.0) containing 1 mM EDTA, in place of diethylene triamine pentaacetic

acid, 1 mM DTT, and 10 mM MgSO_4 . The assay solution consisted of 100 mM Tricine KOH (pH 7.8), 20 mM MgSO_4 , 80 mM glutamate, 4 mM EDTA, 6 mM NH_2OH , 8 mM ATP, plus desalted enzyme in a total volume of 1.0 ml. Samples were obtained at 0 time, and after 5 or 10 minutes incubation at 34°C . Reactions were terminated by adding 1.0 ml of reagent containing 370 mM FeCl_3 , 0.67 N HCl, and 200 mM trichloroacetic acid. The precipitate was removed by centrifugation at 12000 g for 3 minutes and the A_{540} of the supernatant recorded. Blanks were also prepared (mixture minus the enzyme, or without ATP) and amounts of α glutamyl hydroxamate produced were estimated from a standard curve.

Glutaminase

Leaf tissue was ground in 50 mM Tris (pH 8.0), containing 2 mM DTT. Assay mixtures contained 50 mM Tris (pH 8.0), 2 mM DTT, 20 mM glutamine (75 mM stocks of glutamine were passed through Dowex 1, formate form pH 6.8 (Prusiner and Milner 1970), to remove residual glutamate) and desalted enzyme in a total volume of 0.3 ml. Samples were analyzed for either NH_3 or glutamate at 0, and after 60 minutes incubation at 34°C (appropriate controls were run alongside). Aliquots of 0.2 ml were removed for NH_3 determinations by microdiffusion (see below), or 25 mg of 5-sulphosalicylic acid added to reaction mixtures and the resulting supernatant analyzed for the amount of glutamate formed. The amount of glutamate was determined by amino acid analysis.

using sodium citrate buffers. Glutamate was also analyzed by a modified procedure of Curthoys and Lowry (1973). In this case volumes of 0.2 ml were withdrawn from the assay mixture, boiled for 5 minutes, and the protein removed by centrifugation for 3 minutes at 12000 *g*. Aliquots of 0.1 ml were removed from the supernatant and the amount of glutamate assayed enzymatically (see below). Controls containing glutamine or enzyme were treated in the same manner.

2) Analysis of aspartate or glutamate

a) Amino acid analysis (Beckman 119 BL Amino Acid Analyzer)

Aliquots of 0.2 ml were removed from centrifuged enzyme reaction mixtures and brought to pH 2.3 with 1N NaOH. To this mixture, 0.75 ml of lithium-citrate buffer (pH 2.2) was added and 0.4 ml of this solution was loaded onto a Beckman analyzer operating in physiological mode (lithium-citrate buffer, ninhydrin reaction). If the samples were to be analyzed on hydrolysate mode, the pH 2.3 mixture was diluted in sodium citrate buffer, pH 2.3, and 0.4 ml of this solution was loaded for analysis. Lithium-citrate buffers were used for the analysis of aspartate, produced in assays with $(\text{NH}_4)_2\text{SO}_4$ precipitated enzymes obtained from young leaf tissue, since better separations between aspartate and β -(isoxazolin-5-one-2yl)-alanine (X5, Nishikawa and Joy unpublished) resulted. Glutamate, or aspartate (produced in assays with desalted extracts) were analyzed with sodium citrate buffers.

b) Amino acid analysis by HPLC (Waters Analyzer)

Samples of 0.2 ml were removed from assays after acid

precipitation, and 1N NaOH added to bring the pH of the mixture up to about pH 6 (i.e. a solution with reduced buffering capacity). These solutions were diluted with 1.0 ml 10% HPLC grade methanol (in double distilled water), and 10 μ l volumes were removed for derivitization with orthophthaldialdehyde (OPA) and analysis.

c) Enzymatic analysis of glutamate.

A modified method of Curthoys and Lowry (1973) was used to determine glutamate levels in assay mixtures. Enzyme reactions were terminated by boiling for 5 minutes, and proteins removed by centrifugation. Aliquots of the supernatant (0.1 ml) were added to mixtures containing 60 units of glutamate dehydrogenase, 0.03% H_2O_2 (freshly prepared), 0.3 mM ADP, and 1.0 mM NAD^+ . The A_{340} was recorded at 0, and after 20 minutes. Amounts of glutamate were estimated from a standard curve prepared using the above reaction mixture (linear up to 200 nmol glutamate, the highest amount assayed). The amounts of glutamate produced in assay mixtures determined by either amino acid analysis or the modified method of Curthoys and Lowry were equivalent.

3) Analysis of ammonia

a) Nessler's reagent:

Two methods for the preparation of Nessler's reagent were examined, that of Folin and Wu (1965) and of Vaneslow (1940). It was observed that the method of Vaneslow produced less precipitate during preparation and use. The method of Vaneslow was also much easier to prepare than that of Folin and Wu. Vaneslow's procedure involves mixing

22.75 gm of mercuric iodide, and 17.45 gm potassium iodide in as little distilled water as possible (approximately 10-15 ml). An almost saturated solution of KOH (56 gm of KOH added to 70 ml of distilled water and cooled to room temperature) is then added, slowly, to the potassium/mercuric iodide mixture with continuous stirring, and the total volume made up to 500 ml with distilled water. Nessler's reagent was left standing for several days to allow for the precipitation of mercuric iodide prior to use. The reagent was left at room temperature in well stoppered brown bottles.

b) Microdiffusion of ammonia (adapted from Conway and O'Malley (1942) and Burris (1972)):

Aliquots of 0.2 ml were removed from reaction mixtures and added to 1.0 ml of a saturated K_2HCO_3 solution ($pH > 11.0$) in glass scintillation vials. The vials were quickly sealed with rubber stoppers through which fire polished glass rods had been passed and pre-dipped sparingly in concentrated H_2SO_4 . The vials were gently shaken and left for 1 hour (at $22^\circ C$). This time period was ample for the diffusion and entrapment of any NH_3 to take place (fig 1). At the end of this time period the stopper was removed and the rod (with absorbed NH_3) carefully stirred into a mixture of 0.5 ml Nessler's solution and 1.0 ml distilled water. The A_{420} was recorded after 5 minutes. Blanks were concurrently run through the same procedure. The Nessler's- NH_3 solution was observed to be stable for up to an hour in sealed test tubes (e.g. 1.5 ml microfuge

Figure 1

Ammonia nitrogen standard curve. (A) Ammonia standards were measured by microdiffusion for 60 minutes followed by nesslerization ($\blacktriangle$) or by direct nesslerization ($\bullet$). (B) Recovery of ammonia standards as a function of time of microdiffusion. Microdiffusion vials contained 50 ($\blacklozenge$), 100 ($\blacktriangle$), 150 ($\blacksquare$), and 200 ($\bullet$) nmoles of ammonia. Values represent mean $\pm$ SD (n=3). The wavelength maximum of Nessler's reagent, prepared by the method of Vaneslow (1949), was found to be A_{420} .

tubes). Values of NH_3 were obtained from a standard curve, linear up to 400 nmol NH_4Cl (highest amount assayed), using the same procedure as above. A standard curve, prepared by adding NH_4Cl directly to Nessler's, indicated that all of the NH_3 present in the vials was recovered by microdiffusion (fig 1).

4) Protein determinations (Bradford 1976):

Reagent was prepared by mixing 50 mg Coomassie Brilliant Blue G 250 in 25 ml 95% ethanol, followed by 50 ml of 85% phosphoric acid. The volume was made up to 500 ml with distilled water, mixed well, and filtered. This solution was stable at room temperature for at least a month. Protein samples of 0.1 ml were mixed with 5.0 ml of reagent and left from 5-30 minutes prior to determining the A_{595} . Standard curves were prepared with bovine γ globulin, and were linear only up to 80 μg protein. Desalted leaf extracts were diluted on average 20 fold and $(\text{NH}_4)_2\text{SO}_4$ preparations were diluted on average 50 fold prior to protein determinations.

5) Analysis of leaf amino acid levels by HPLC:

Leaf tissues were ground in 3 volumes of water or buffer and filtered through 4 layers of premoistened cheese cloth. The macerates were centrifuged at 20000 g for 10 minutes. Aliquots of 0.3 ml were removed from the supernatant and proteins were precipitated with

5-sulfosalicylic acid. Samples were removed from the second supernatant, neutralized with 1N NaOH (pH 6-7), and diluted with 10% HPLC grade methanol, so that the 10 μ l volume analysed was equivalent to 0.3 mg of the original fresh weight.

6) Analysis of xylem sap

a) Sap collection

Soil grown plants were well watered prior to stem excision, while hydroponic plants had no special treatment. On average about 40 plants were used for each xylem sap collection. Stems were cut just above the cotyledons (or the cotyledonary scar in hydroponic plants) and the severed stems were rinsed with distilled water. Xylem exudates were collected from actively bleeding roots over a 30 minute period using capillary tubes.

b) Amino acid analysis of root exudates by HPLC:

Aliquots of 0.05 ml were diluted 10 fold with 10% HPLC grade methanol, and 10 μ l volumes sampled for analysis.

c) NO₃⁻ analysis of root exudates (procedure of Johnson and Ulrich 1950)

Aliquots of 0.05 ml were removed from collected exudates and dried under a heat lamp. The samples were cooled, 1.0 ml phenoldisulphonic acid added, and mixed well. To these mixtures 4.0 ml of distilled water was added, then the solutions were stirred well, cooled, and 8.0 ml of 33% (v/v) NH₄OH was added. After cooling, the volumes were made up to

20.0 ml and the A_{408} determined. A standard curve was prepared with KNO_3 , and was linear up to 200 μg . KNO_3 , the highest amount tested.

7) Column chromatography

a) Ion exchange:

Dowex 1 Cl^- form was converted to the acetate form prior to use. The resin was suspended in 1N NaOH, and repeatedly rinsed with distilled water to neutral pH. This was followed by resuspending the resin in 2N HCl, and repeatedly washing with distilled water until neutral. A 10% Na_2CO_3 solution was then added and the resin washed with distilled water. Finally 2N acetic acid was added to the resin, followed with repeated distilled water washes to neutral pH. Dowex 1 was then equilibrated with 50 mM Tris HCl, pH 6.8, before the addition of asparagine or glutamine solutions.

DEAE cellulose was suspended in 0.25 N NaOH, and washed with distilled water until neutral. This was followed by resuspension in 0.25N HCl and 0.25 N NaOH with water washes between, and finally brought to pH 8.0 with buffer.

DEAE cellulose columns (1 cm. X 26 cm.) were packed at flow rates of 30 ml/hr. and run at 20 ml/hr. Prior to use columns were equilibrated in extraction buffer. Desalted leaf extracts, on average 20 ml volumes (approx. 8 gm. fresh weight), were loaded onto columns and followed with 80-100 ml washes with extraction buffer. Gradients of 0.05-0.2 M KCl (200 ml) were applied to the columns using a Pharmacia gradient maker

61
and 2.0 ml fractions were collected. Each fraction was assayed for protein (A_{280}), enzyme activity, and conductance. Conductance was measured on a Yellow Springs conductivity meter. KCl concentrations, in the column eluants, were estimated from a standard curve (conductance vs KCl concentration).

b) Gel permeation chromatography (Sephacryl S 300):

The gel was suspended in de-aerated extraction buffer, packed at 30 ml/hr., and run at 20 ml/hr. Prior to use, columns (2.6 cm. X 80 cm.) were equilibrated in extraction buffer. Leaf extracts (approx. 8 gm fresh weight) were harvested in 1.0 volumes of buffer and centrifuged at 20000 *g*. The resuspended precipitates, from 30-65% saturation with $(\text{NH}_4)_2\text{SO}_4$, were loaded onto the column. Fractions of 5.0 ml were collected and assayed for protein at A_{280} , and enzyme activity. The relative molecular mass (M_r) of asparaginase was determined from S300 columns calibrated with Blue Dextran (M_r 200,000), Alcohol dehydrogenase (M_r 150,000), Bovin serum albumin (M_r 66,000), Carbonic anhydrase (M_r 29,000), and Cytochrome C (M_r 12,400).

8) Feeding experiments:

Just before the end of the dark period, whole shoots were excised above the cotyledon and quickly transferred to distilled water. Petioles of half expanded leaves were cut under water (to maintain an intact xylem stream), just above the stipules, and placed individually in small tubes containing feeding solutions. Unless otherwise stated, the

solutions were at pH 6.3 and contained 8 mM asparagine (passed through Dowex 1), together with other additional materials. Samples were also taken for immediate determination of asparaginase activity at the end of the dark period (0 hour). Usually 6 treatments, including a control, could be carried out per experiment, with 10 replicates per treatment. Trays of treated half expanded leaves were placed in the light, in growth cabinets, for periods of 2.5-4 hours. The solutions were topped up every 1/2 hour or so as needed throughout the experiment.

9) Estimation of photosynthetic activity

a) DCPIP reduction (see Izawa 1980):

Leaf material was ground in 50 mM potassium phosphate buffer, pH 8.0, containing 10% glycerol, 50 mM KCl, 0.2 M sucrose and chloroplast membranes were sedimented at 20000 *g* for 10 minutes. The pellet was resuspended in 50 mM potassium phosphate buffer, pH 8.0, 10% glycerol, 50 mM KCl, 5.0 mM $MgCl_2$ and diluted to a concentration of 10-15 mg chlorophyll/l. Chlorophyll concentrations were determined by the procedure of Wintermans and Demots (1965). Assays were conducted in 7.0 ml cuvettes containing resuspension buffer (at 22°C), 35 μ M DCPIP, and 5-8 μ g. chlorophyll, in a total volume of 6.0 ml. The reactions were started by adding the extract, mixing well, and recording the initial optical density (A_{610} approx. 0.7). The tubes were then placed before a 300 Watt incandescent light source. A water heat-filter was placed between the light source and the assay mixtures. The decrease in A_{610}

was recorded over a 10 minute period.

b) O_2 evolution from chloroplast membranes (adapted from Lilley et al. 1975):

Leaf samples were ground in 50 mM potassium phosphate buffer, pH 8.0, containing 10% glycerol, 0.2 M sucrose, and 50 mM KCl. Membranes sedimented at 20000 *g* (10 minutes) were resuspended in 50 mM potassium phosphate buffer, pH 8.0, 10% glycerol, 0.2 M sucrose, 50 mM KCl, 5 mM $MgCl_2$ and diluted to 500 mg chlorophyll/l (chlorophyll determination by the method of Wintermans and Demots, 1965). Assays involved the addition of 60-80 μg chlorophyll to a vial containing resuspension buffer, 2.0 mM $K_3Fe(CN_6)$, and 4.0 mM NH_4Cl (all at 22°C) in a total volume of 4.0 ml. The O_2 probe was inserted into the vial, the mixture allowed to equilibrate for approximately 3 minutes in the dark, and the assay started by illuminating the vial with light from a halogen lamp (slide projector), passed through a water heat-filter. The evolution of O_2 was measured with a Yellow Springs O_2 meter.

c) O_2 evolution from leaf pieces (see Dillieu and Walker 1981):

Leaf material was weighed and briefly dipped in a solution containing 50 mM potassium phosphate, 10% glycerol, 50 mM KCl, 50 mM Mg_2Cl , and 0.1% Triton X 100 (at 22°C). Leaves ranging from 0.08 to 0.1 gm original fresh weight were cut on a chilled glass plate into strips 2.0 mm. wide and added to assay solutions containing 50 mM potassium phosphate, 10% glycerol, 50 mM KCl, and 10 mM $NaHCO_3$ at 22°C. The O_2 probe was

inserted into the vial and the mixture left to equilibrate for 3 minutes in the dark. The preparation was then illuminated (slide projector, halogen light source, passed through a water heat-filter) for 3 minutes, and again darkened for a 3 minute period; this cycle was repeated for 4 trials. The O_2 evolved (and consumed in the dark) was determined using a Yellow Springs O_2 meter. The O_2 probe was calibrated before and after a series of experiments, using a known volume of air saturated buffer (at $22^\circ C$) and by removing all the O_2 present with $Na_2S_2O_4$ (sodium dithionite). Prior to the addition of $Na_2S_2O_4$, the initial output from the probe was noted for a 4 ml volume of air saturated buffer, the dithionite was then added, the mixture stirred well and the final output recorded as '0' O_2 . The difference in the two outputs on the chart recorder was measured and calibrated to correspond to $250\mu M$ (estimated O_2 concentration obtained from a table of O_2 concentrations in aqueous solutions, Leegood et al. 1984).

d) In vivo fluorescence determination (see Baker and Bradbury 1981, Papageorgiou 1975):

In vivo chlorophyll *a* fluorescence was estimated using a Plant Productivity Fluorometer (Model SF-20 Brackner and Assoc. Ottawa). Leaf material was carefully placed across the probe of the instrument, avoiding midrib sections, and dark adapted for 5 minutes. This tissue was then illuminated at 670 nm (4×10^3 ergs/cm²/sec) for a period of 100 seconds. Over this time period the fluorescence at 685 nm (F_{685})

was followed with a chart recorder. At the end of a trial, relative values for 'O', 'P', and 'T' (intermediates along the fluorescence induction curve) were obtained from the fluorometer.

The initial increase in fluorescence, 'O-P', along the fluorescence induction curve represents the reduction of the plastoquinone pool (PQ pool). As electrons flow from the PQ pool towards photosystem I the fluorescence yield decreases and passes through several intermediates, 'S' and 'M', before reaching steady state levels at 'T' (Bradbury 1981). The addition of DCMU or atrazine (compounds that block electron transport between the primary electron acceptor 'Q' and PQ, Trebst 1980) results in the maintenance of a high level of fluorescence between the 'P-T' transients of the fluorescence induction curve.

10) Analysis of radioactivity in leaf homogenates:

Leaf samples were homogenized in 3 volumes of extraction buffer. Aliquots of 0.3 ml were removed from this initial macerate, brought to 80% ethanol (v/v), and centrifuged for 3 minutes at 12000 *g*. The pellet was solubilized in either 0.4 ml of NCS tissue solubilizer (followed with heating at 45°C for 1 hour), or 0.2 ml of 1N NaOH. In both cases the mixtures were left with occasional stirring at room temperature until dissolved. The suspensions were adjusted to pH 6 with concentrated glacial acetic acid, and aliquots (0.1 ml) were added to 5 ml TOX scintillant and counted for 10 minute periods per sample. TOX scintillant was prepared by mixing 4.0gm Omnifluor (New England Nuclear), in 750 ml Xylene, followed by the addition of 250 ml Triton X114. Radioactivity was determined using a Beckman Scintillation

counter (model L37500).

Results

I. Activity of asparaginase from developing pea leaves

I.1 Plant and leaf development

When plants were grown under the conditions stated in the methods section, developing first leaves were observed to be half expanded 8 days after imbibition. Anthesis was observed 22-23 days after imbibition and at this age 7-8 leaves were present on the plants with leaf number 7 (counting from the bottom) in the half expanded state. Plants grown hydroponically or in soil developed at the same rate. Hydroponically-grown plants, at the same age and leaf number as soil grown plants, were much larger and yielded higher fresh weights on a per leaf basis than soil grown plants (for differences in asparaginase activity between hydroponic and soil plants see sections I.10, III.1, and III.12). For most experiments in this study leaf material was obtained from 15-18 day old plants. These plants had 4 expanded leaves and a developing fifth leaf. The developing fifth leaf was routinely used as a source of material for enzyme analysis.

P. sativum leaves pass through a series of 5 morphologically distinct stages during leaf development which have been described and previously characterized (see Urquhart and Joy 1982, and figure 2). A young leaf is one that is just emerging from within the stipule of the next older leaf and this stage of development is referred to as stage 3; a stage 5 leaf is one that is in the half expanded state. Since leaves of both hydroponically and soil grown plants passed through successive leaf stages at approximately 1 day intervals, stage 3 leaves developed into half expanded, stage 5 leaves over a 2 day period.

Figure 2

Stages and age of developing pea leaves. Developing pea leaves pass through a series of distinct morphological stages each approximately one day apart. Age of a leaf (L) is measured in terms of days after emergence from stipule (S) of the next older leaf. (figure is modified from Urquhart and Joy 1982)

stage

age in
days

7

5

6

4

5

3

4

2

3

1

2

0

1

0

1.2 Asparaginase extraction and stability

Young leaves, at stage 3, were examined as a source for asparaginase since stage 3 leaves were reported to have high levels of activity (Ireland and Joy 1982). Leaf material was routinely extracted and assayed for asparaginase activity using either 50 mM Tris HCl containing 50 mM KCl, or 50 mM potassium phosphate containing 10% glycerol, and similar activities were obtained with either buffer. Equivalent asparaginase activities were also observed with 50 mM Hepes, Epps, Tricine or Tris, all at pH 8.0. When stage 3 leaf tissue was extracted in Tris HCl buffer a loss in asparaginase activity was observed upon storage at 4°C over a 2 day period (figure 3; only a marginal loss of asparaginase activity was observed over a 6 to 8 hour period when kept on ice). The addition of DTT to the crude enzyme extract extended survival by a day. In comparison, asparaginase obtained from developing seed tissue, 12-15 days after anthesis, was stable up to 4-5 days (figure 3). The stability of asparaginase, obtained from leaf tissue was marginally protected with K⁺. The addition of K⁺ to extracts increased *in vitro* asparaginase activity (see figures 24 and 25). Incubation experiments (see next section) performed with leaf extracts at 34°C demonstrated that asparaginase activity was more stable in potassium phosphate buffer containing 20% glycerol than in Tris HCl KCl (with 2 mM DTT) (see table 13), however no differences in extractable activity were observed in asparaginase, obtained in potassium phosphate containing 10% glycerol or Tris HCl KCl (±DTT) buffer. Based on these observations, asparaginase was routinely extracted and assayed in either Tris HCl KCl (±DTT), or potassium phosphate buffer with 20% glycerol added. Unless otherwise noted, asparaginase activity was determined as soon as possible, usually 30 to

Figure 3

Stability of asparaginase during storage at 4°C. Young (stage 3) leaves were harvested in 50 mM Tris HCL buffer, pH 8.0, containing 50 mM KCl and 2 mM DTT (○), or Tris HCl buffer containing only DTT (■), or only KCl (●) and stored at 4°C. Aliquots were removed and assayed for asparaginase activity at the times indicated. The stability of asparaginase extracted from developing pea cotyledons (▲) in the presence of KCl and DTT is presented for comparison. Values are expressed as percent of original (day 1) activity.

Asparaginase

- ▲—▲ seed KCl DTT
- young leaf +KCl DTT
- - DTT
- - KCl

45 minutes after extraction, to minimize any alterations in activity due to instability in storage.

1.3 Asparaginase inactivation

To examine the effects of compounds on asparaginase activity, experiments were performed that involved preincubating crude preparations of asparaginase for 30 minutes at 34°C, along with the compound of interest before carrying out the assay (a further 30 minutes). Half expanded leaves were harvested at the end of the dark period or after 6 hours in the light and desalted in 50 mM Tris HCl, pH 8.0, containing 50 mM KCl and 1 mM CaCl₂. Asparaginase activity was assayed at the start of the incubation period (initial activity), and after 30 minutes incubation. Controls containing enzyme, without additions, were run alongside so that asparaginase activity in the presence or absence of additions could be compared.

Asparaginase activity (assayed in 20 mM asparagine) was stable and the reaction was linear for 90 minutes (i.e. over 3 times the length of the incubation treatment, longest period tested), although when incubated for 30 minutes in Tris KCl CaCl₂ buffer without substrate, asparaginase was not stable. Activity decreased over the first 15-20 minutes of incubation in the absence of substrate (figure 4). This indicated that asparagine may help stabilize asparaginase. To study this relationship in more detail asparaginase was incubated with a range of substrate concentrations (0-10 mM asparagine) for 30 minutes prior to assay in a final concentration of 20 mM asparagine. In 10 mM asparagine, asparaginase was stable and no loss in activity was observed when compared with initial activity, while at 4 mM asparagine only 60% of the original activity

Figure 4

Stability of asparaginase at 34°C. Half expanded (stage 5) leaves were harvested at the end of the dark period (●) or after 6 hours in the light (○) in 50 mM Tris HCl, pH 8.0, containing 50 mM KCl and 1 mM CaCl_2 .

Extracts were incubated for up to 60 minutes at 34°C before asparaginase activity was assayed. At the indicated times, an aliquot was removed from the incubating extract and asparagine added to start the reaction. A 0 minute sample was obtained at the start of the assay and activity was corrected for background aspartate levels.

Asparaginase activity is expressed as: nmol asp/min/gm fr. wt.

was noted in the assay following the 30 minute incubation. On average, in the absence of asparagine, 40-45% of the original activity was recovered. (figure 5). Aspartate or NH_3 , products of the asparaginase reaction, were also added to asparaginase preparations during a 30 minute incubation and aspartate was found to protect asparaginase activity while NH_3 had no effect during incubation (figure 5).

During incubation experiments (see section III.2), 4 mM asparagine was routinely added to treatments along with compounds to be tested to partially stabilize asparaginase. Aspartate was not added in place of asparagine during incubation experiments since asparaginase activity was determined by measuring aspartate levels.

1.4 Diurnal variation of asparaginase activity in young leaves

Analysis of asparaginase activity obtained from stage 3 leaf tissue indicated that extractable asparaginase activity varied during the day. When assayed over a 24 hour period, asparaginase activity from stage 3 leaves increased steadily through the photoperiod (expressed on a fresh weight basis, figure 6). In the dark period the levels decreased to those observed at the onset of the photoperiod. These experiments (figure 6) were performed upon nodulated plants; a similar trend was observed in nonnodulated plants grown in soil or hydroponically grown. The diurnal change in extractable activity was also observed when activity was expressed on a per mg. protein basis, or on a per leaf basis. The increase in activity during the light period was not due to the difference in temperature between periods of dark (18°C) and light (25°C). When the temperature was increased to 25°C after the dark period and the plants

Figure 5

Effect of asparagine, aspartate and ammonia on the stability of asparaginase. Half expanded (stage 5) leaves were harvested after 6 hours in the light and incubated in varying concentrations of asparagine (●), aspartate (▼), and ammonia (■) for 30 minutes at 34°C. After the incubation period asparaginase activity was assayed by adding asparagine to the incubating mixtures to start the reaction. A 0 minute sample was removed at the start of the assay and activity was corrected for background aspartate levels. Initial asparaginase activity of the enzyme preparation, before incubation, was: 100 nmol asp/min/gm fr wt

Figure 6

Diurnal variation of asparaginase activity in young leaves. Asparaginase activity (Δ) and leaf asparagine levels ($\circ$) were monitored in young (stage 3) leaves from soil grown plants. Asparaginase activity is expressed as nmol asp/min/gm fr wt, and values represent mean $\pm$ SD (n=3).

2

MICROCOPY RESOLUTION TEST CHART
NATIONAL BUREAU OF STANDARDS
STANDARD REFERENCE MATERIAL 1010a
(ANSI and ISO TEST CHART No. 2)

maintained in the dark, a steady decrease in asparaginase activity was still observed (see figure 15).

1.5 Diurnal variation of amino acid and ammonia levels in young leaves

The levels of asparagine and several other amino acids in stage 3 leaf tissue were monitored over a 24 hour period (figs 6, 7). Asparagine levels decreased in the light and increased in the dark, compared with asparaginase activity which increased in the light and decreased in the dark (figure 6). The variations in glutamine, homoserine and aspartate over the photoperiod are shown in figure 7, and no trend seems apparent. Glutamate was observed to fluctuate throughout the photoperiod in this and other experiments, but no consistent trend in levels was noted. The levels of ammonia in young leaf tissue remained low (1-1.5 $\mu\text{mol/gm. fr. wt.}$) with only slight variation throughout the day (figure 8).

1.6 Diurnal changes of related enzymes of nitrogen metabolism

Since the variation of asparaginase could result in a diurnal variation in ammonia release, it was of interest to look for daily changes in the activity of glutamine synthetase which would be responsible for ammonia reassimilation. When glutamine synthetase, extracted from stage 3 leaves, was analyzed over a 24 hour period no consistent diurnal pattern was evident. Extractable activity did fluctuate, but from day to day there was no consistent time for a peak of activity (figure 8). The activity of glutamate dehydrogenase (NADH-GDH; amination of oxoglutarate) obtained from stage 3 leaves was much lower than that of glutamine synthetase (maximal NADH-GDH activity: 62.3 nmol./min/gm.fr.wt. vs. maximal GS activity: 411.0 nmol./min/gm.fr.wt.). NADH-glutamate dehydrogenase

Figure 7

Diurnal changes of several amino acids in young leaves. Young (stage 3) leaves were harvested from soil grown plants at the times indicated and amino acids levels ($\mu\text{mol/gm fr. wt.}$) determined. glutamine (Δ), glutamate (o), homoserine ($\square$), aspartate ($\bullet$), and asparagine ($\blacksquare$).

Figure 8

Diurnal changes in glutamine synthetase activity and ammonia levels in young leaves. Young (stage 3) leaves were obtained from soil grown plants. Glutamine synthetase activity (expressed as nmol α -glutamyl hydroxymate/min/gm fr wt.) ($\bullet$) and ($\blacktriangle$) were assayed during two independent experiments. Note that the axis for ammonia levels ($\square$) is half of that in figure 7

activity varied throughout the photoperiod and decreased during the light period (figure 9). When assayed in the reverse direction (oxidative deamination of glutamate), no NAD^+ -glutamate dehydrogenase activity was detected in stage 3 leaf tissue during the light period. The activity of glutaminase was also observed to decrease in the light period (figure 9), although this change in activity was not as pronounced as the increase observed in asparaginase activity during the same period. Asparagine aminotransferase did not appear to undergo any marked diurnal variation in activity. Activity at the end of the dark period, obtained from stage 3 leaf extracts, was 9.0 nmol./min./gm. fr. wt. compared with 6.2 nmol./min./gm. fr. wt. for extracts obtained at the end of the light period. In half expanded (stage 5) leaves, asparagine aminotransferase activities of 36.2 and 47.0 nmol./min./gm. fr. wt. were recovered in extracts harvested at the end of the dark and light periods respectively.

1.7 Asparaginase activity and leaf age

Asparaginase has been reported to decrease with leaf age (Ireland and Joy 1981), therefore the increase in activity over the light period was investigated throughout the development of leaf number 5. Leaf tissue was harvested at five different ages and examined for asparaginase activity at the end of the light and dark periods (figure 10). During development, there was a steady decrease in asparaginase activity when measured at the end of the dark period. However, activity measured at the end of the light period showed a different relationship. Maximum activity first increased, then remained high during the early stages of development, and finally fell rapidly (figure 10). The greatest stimulation in activity by light was observed in half expanded leaves (stage 5),

Figure 9

Diurnal changes in glutamate dehydrogenase and glutaminase activities in young leaves. Young (stage 3) leaves were obtained from soil grown plants. Glutamate dehydrogenase activity ($\blacktriangle$) is presented as nmol NAD⁺/min/gm fr. wt., and glutaminase activity ($\bullet$) as nmol glu/min/gm fr. wt.

Figure 10

Diurnal variation of asparaginase activity during leaf development.

Leaves from soil grown plants were harvested at the end of the dark (●) or light (○) periods and assayed for asparaginase activity (expressed as nmol asp/min/gm fr wt). Values represent mean $\pm$ SD. (n=3). Leaf age is measured in days after emergence from stipule (see figure 1)

approximately 3 days after emergence from the stipule and ranged from a 3-5 fold increase in activity (see also table 1). Thus the observed pattern of asparaginase activity during development depends on the time of day used in a sequence of measurements (e.g. dotted lines figure 10). Very young leaves (stages 1-2, younger than that used for earlier experiments, figure 6), or mature leaf tissues (stages 7-8) did not exhibit a diurnal change in extractable activity. This pattern of activity with leaf age was observed in both nodulated and nonnodulated plants.

The diurnal variation of asparaginase activity was examined in half expanded leaves since the increase in asparaginase activity was most noticable in leaves at this stage of development (figure 11). Asparaginase activity continued to decrease over the first hour of light exposure. This loss in activity was followed by an increase in activity over the following 2 hours, then asparaginase activity was maintained at a high level for the duration of the light period (figure 11). On transfer to darkness, asparaginase activity remained high for about 1 hour before showing a rapid decline (figure 11). The changes in asparaginase activity appeared to be more rapid than in the leaf at stage 3 (figure 6).

1.8 Asparaginase, leaf amino acid levels, xylem exudates and plant age

Extractable asparaginase activities in successive leaves at stage 3, and the corresponding stage 5 leaves on the same plant, were examined over the vegetative life cycle of soil grown, nonnodulated plants from 8 to 22 days after imbibition. Samples of the first through to the eighth leaf were taken at 2 developmental leaf stages so that asparaginase activity could be compared with leaf number as the plant developed. The samples were harvested at the end of the dark period and after 6 hours in the light

Figure 11

Diurnal variation of asparaginase activity in half expanded leaves. Half expanded (stage 5) leaves were obtained from soil grown plants. Asparaginase activity expressed as nmol asp/min/gm fr wt, values represent mean $\pm$ SD (n=3).

and assayed for asparaginase activity (table 1), as well as for endogenous amino acid levels (figure 12, tables 2 & 3). Xylem exudates collected from root stumps of decapitated plants were also collected at the end of the dark period and after 6 hours of light (figure 13, table 4).

Extracts of stage 3 or half expanded leaves (stage 5) obtained from leaf numbers 1 to 3 displayed little diurnal variation in asparaginase activity (table 1). Greater increases in asparaginase activity during the 6 hour light period were observed in both young and half expanded leaves of older plants (extracted from leaves 4 through to 8, taken 15 to 22 days after imbibition). This trend was observed when activities were analyzed on a fresh weight basis (table 1), or on a mg protein-basis. Activity of asparaginase harvested at the end of the dark period was greater in stage 3 leaf tissue compared with that obtained from half expanded leaves (stage 5) over the 8 to 22 day period (table 1). This is consistent with the trend observed in developing fifth leaf extracts from 15 to 18 day old plants (figure 10).

Amino acids were analyzed from homogenates of young (table 2) and half expanded leaves (table 3) at the end of the dark period, and also after 6 hours of light. The levels of asparagine, aspartate, glutamine, glutamate, and homoserine are illustrated in figure 12 so that the trends observed in asparagine can be compared with those of the more abundant amino acids (note also arginine and serine, tables 2 & 3). In general, levels of most leaf amino acids remained relatively constant in successive leaves at the same stage of development and were slightly lower after 6 hours in the light. The only pronounced change was found in the levels of asparagine (see figure 12) which decreased from leaf 1 to leaf 8. Levels of asparagine were also consistently lower in leaf extracts obtained

Table 1

Changes in asparaginase activity in young and half expanded leaves during plant growth from 8 to 22 days. Successive young (stage 3) and half expanded leaves (stage 5) were assayed for asparaginase activity at the end of the dark period (0hr.), and after 6 hours exposure in the light, from 8-22 days after imbibition. The % increase (%inc.) in activity during the 6 hour light period is also listed.

<u>stage 3 leaves</u>					<u>half expanded leaves.</u>				
Plant age in days	leaf #	<u>n mol/min.</u> gm.fr.wt.		% inc.	Plant age in days	leaf #	<u>n mol/min.</u> gm.fr.wt.		% inc.
		0 hr	6 hr.				0 hr.	6 hr	
		(not assayed)			8	1	3.5	4	37
8	2	6.4	8.5	33	11	2	6.0	20.7	245
11	3	14.1	39.8	182	13	3	6.6	21.6	272
13	4	39.4	59.0	50	15	4	10.3	53.7	421
15	5	37.0	55.8	51	18	5	12.2	75.6	520
18	6	40.3	99.4	147	20	6	14.7	86.1	486
20	7	33.4	78.1	134	22	7	18.1	70.3	288
22	8	48.7	101.0	107					

Figure 12

Amino acid levels in successive young and half expanded leaves during plant development. Successive young (stage 3) and half expanded (stage 5) leaves were taken from developing plants 8 to 22 days after imbibition. Samples were obtained at the end of the dark period (solid symbols) and after 6 hours in the light (open symbols). Asparagine (o), aspartate (Δ), glutamine ($\square$), glutamate ($\diamond$), and homoserine (∇). For complete amino acid levels in young and half expanded leaves see tables 2 and 3.

Table 2

Amino acid levels in stage 3 leaves. Successive stage 3 leaves were taken from developing plants 8 to 22 days after imbibition. Samples were obtained at the end of dark period (0 hr.) and after 6 hours in the light, ground, centrifuged and an aliquot removed from the supernatant for amino acid determinations. Total α -amino nitrogen levels (total) are presented below each leaf sample.

$\mu\text{mol/gm. fr. wt}$								
Leaf #	2		3		4		5	
Plant age	8		11		13		15	
	0 hr	6 hr	0 hr	6 hr	0 hr	6 hr	0 hr	6 hr
Asn	18.5	13.7	17.9	24.0	15.1	6.2	10.8	10.8
Asp	2.6	2.7	3.5	3.1	4.5	1.3	4.0	3.0
Gln	7.4	5.8	3.2	2.0	3.0	1.4	3.9	2.7
Glu	5.8	6.3	5.9	5.2	4.8	2.3	6.4	4.6
Hse	4.6	4.4	1.8	1.8	0.5	0.7	1.3	1.2
Gly	0.11	0.13	0.08	0.09	0.09	0.07	0.12	0.1
Ser	2.2	1.5	1.5	1.09	1.15	0.65	1.5	1.1
Ala	0.9	1.0	0.8	0.98	0.62	0.43	0.9	0.9
Arg	2.6	2.6	1.4	1.4	0.39	0.32	0.33	0.6
Thr	0.52	0.48	0.35	0.35	0.35	0.27	0.4	0.3
Tyr	1.8	1.6	0.67	0.56	0.56	0.35	0.6	0.6
His	tr.	tr.	3.2	2.8	3.1	1.2	1.6	1.8
Met	0.12	0.11	0.1	0.43	0.1	0.06	0.09	0.1
Val	0.65	0.53	0.57	0.58	0.37	0.17	0.44	0.4
Phe	0.81	0.77	0.79	0.81	0.12	0.1	0.13	0.3
Ile	0.16	0.15	0.13	0.13	0.11	0.05	0.15	0.12
Leu	0.12	0.09	0.07	0.05	0.05	0.05	0.08	0.08
Lys	1.26	0.91	0.75	0.69	0.28	0.3	0.27	0.21
Total	50.2	42.2	42.7	46.1	35.2	16.2	30.0	28.9

continued...

Table 2 continued
 Amino acid levels in stage 3 leaves
 $\mu\text{mol/gm fr. wt.}$

leaf #	6		7		8	
Plant age:	18		20		22	
	0 hr.	6 hr.	0 hr.	6 hr.	0 hr.	6 hr.
Asn	4.62	6.3	3.1	2.15	1.88	1.4
Asp	2.71	2.6	2.31	1.3	1.26	1.21
Gln	2.4	2.53	2.02	1.93	1.99	2.18
Glu	5.6	5.1	5.53	2.66	2.81	2.67
Hse	0.85	1.46	0.96	0.67	1.18	1.32
Gly	0.09	0.12	0.08	0.05	0.09	0.07
Ser	1.31	1.21	1.38	0.7	0.98	0.99
Ala	0.83	0.89	0.77	0.43	0.38	0.37
Arg	0.1	0.31	0.1	0.09	0.1	0.13
Thr	0.31	0.47	0.37	0.24	0.4	0.41
Tyr	1.12	1.11	1.67	0.92	tr.	0.48
His	0.87	0.96	0.75	0.22	tr.	tr.
Met	0.09	0.12	0.09	0.06	0.06	0.05
Val	0.48	0.42	0.42	0.23	0.19	0.24
Phe	0.1	0.18	0.1	0.08	0.05	0.07
Ile	0.13	0.12	0.11	0.06	0.05	0.07
Leu	0.08	0.06	0.07	0.06	0.07	0.1
Lys	0.16	0.19	0.13	0.17	0.13	0.13
Total:	21.9	24.2	20.0	12.0	11.6	11.9

Table 3

Amino acid levels in half expanded leaves. Successive half expanded leaves (stage 5) were taken from developing plants 8 to 22 days after imbibition, at the same time as young leaf samples (table 2). Samples were obtained at the end of dark period (0 hr.) and after 6 hours in the light, ground, centrifuged and an aliquot removed from the supernatant for amino acid determinations. Total α -amino nitrogen levels (total) are presented below each leaf sample.

$\mu\text{mol/gm fr. wt}$								
Leaf #:	1		2		3		4	
Plant age:	8		11		13		15	
	0 hr.	6 hr.	0 hr.	6 hr.	0 hr.	6 hr.	0 hr.	6 hr.
Asn	16.5	14.3	28.1	29.8	14.3	8.96	11.0	10.9
Asp	2.5	2.3	3.0	2.1	2.7	0.86	2.8	2.27
Gln	8.2	4.9	2.7	1.3	2.37	1.02	3.1	2.1
Glu	6.8	7.9	5.4	5.0	3.87	1.94	6.0	4.7
Hse	3.7	3.5	1.7	1.47	1.02	0.72	1.2	1.1
Gly	0.12	0.11	0.09	0.1	0.07	0.06	0.1	0.1
Ser	2.5	1.7	1.6	1.4	1.01	0.7	1.3	1.91
Ala	1.83	1.07	0.85	0.98	0.52	0.49	0.89	1.0
Arg	3.7	2.7	2.5	2.6	0.58	0.67	0.1	0.1
Thr	0.43	0.44	0.33	0.3	0.27	0.18	0.25	0.25
Tyr	1.37	1.28	0.49	0.27	0.21	0.23	0.37	0.32
His	tr.	tr.	2.8	3.04	2.36	1.01	1.5	1.8
Met	0.1	0.1	0.1	0.13	0.08	0.06	0.08	0.09
Val	0.57	0.45	0.52	0.4	0.33	0.15	0.31	0.24
Phe	0.38	0.38	0.36	0.41	0.07	0.08	0.08	0.13
Ile	0.18	0.15	0.19	0.12	0.14	0.07	0.15	0.12
Leu	0.11	0.08	0.1	0.05	0.07	0.06	0.07	0.04
Lys	1.5	1.17	1.15	1.18	0.36	0.46	0.26	0.27
Total:	40.5	42.5	52.0	50.6	30.0	17.7	29.6	27.4

continued...

Table 3 continued.
 Amino acid levels in half expanded leaves.
 $\mu\text{mol/gm fr. wt.}$

Leaf #	5		6		7	
Plant age	18		20		22	
	0 hr.	6 hr.	0 hr.	6 hr.	0 hr.	6 hr.
Asn	7.4	5.8	2.7	2.2	2.3	1.8
Asp	3.4	3.1	1.1	0.89	0.8	1.1
Gln	3.47	2.1	1.2	1.6	1.3	1.9
Glu	7.2	6.1	2.8	2.7	2.3	2.7
Hse	1.3	2.0	0.56	0.54	1.08	1.37
Gly	0.09	0.08	0.05	0.04	0.05	0.07
Ser	1.74	3.29	0.67	0.56	0.6	0.76
Ala	1.08	1.79	0.41	0.45	0.28	0.6
Arg	0.14	0.18	0.13	0.13	0.02	0.05
Thr	0.32	0.25	0.17	0.18	0.24	0.34
Tyr	0.71	0.78	0.61	0.42	tr.	tr.
His	tr.	1.1	tr.	0.05	tr.	tr.
Met	0.09	0.29	0.06	0.06	0.04	0.07
Val	0.4	1.14	0.15	0.13	0.1	0.15
Phe	0.1	0.63	0.04	0.06	0.04	0.11
Ile	0.16	0.63	0.06	0.06	0.05	0.06
Leu	0.1	0.09	0.08	0.06	0.05	0.07
Lys	0.21	0.83	0.19	0.18	0.08	0.16
Total:	27.9	30.2	11.0	10.3	9.3	11.3

during the light period (except for extracts obtained from 11-day-old young and half expanded leaves, where the levels in the light were greater than those at the end of the dark period) and this trend was more apparent in young leaf tissue.

Xylem exudates were also collected at the end of the dark period and after 6 hours in the light (table 4, figure 13) from plants 11 to 22 days old. Root stumps of 8 days old plants did not bleed, possibly a result of insufficient root pressure since their root systems were not well developed as yet, and xylem sap was not obtained. The concentration of total organic nitrogen decreased as the plants developed (figure 13a). The most dramatic change was observed over the 11 to 15 day period after imbibition. Changes in the concentrations of the total organic nitrogen were mostly accounted for by the decreases observed in asparagine and homoserine levels (table 3, figure 13c). Aspartate and glutamate were the only two amino acids that did not appear to vary during plant development. Over the 6 hour light period a reduction in the concentration of total organic nitrogen was seen in xylem exudates obtained from young excised plants 11 to 15 days old. The diurnal variation in amino acid concentrations was much reduced in mature plants (15 to 22 days after imbibition).

When total nitrogen (organic + NO_3^-) was examined, a decrease in concentration with plant age was most pronounced in the exudates collected at the end of the dark period (figure 13b). The concentrations of total nitrogen collected after 6 hours of illumination varied between 10-14 mM and displayed no distinct trend as plants aged. The presence of NO_3^- in exudates increased with plant age, and 13 days after imbibition the

Figure 13

Amino acid and nitrate levels in xylem exudates during plant development. Plant stems were cut just above the cotyledon, rinsed with distilled water, and the exudate collected for a 30 minute period at the end of the dark period (solid symbols) or after 6 hours in the light (open symbols). (a) sum of molarities of organic nitrogen, (b) sum of molarities of organic and nitrate nitrogen, (c) levels of asparagine ($\square$), homoserine ($\circ$), glutamine (Δ), and nitrate ($\diamond$). See table 4 for levels of other amino acids in the xylem exudate

days after imbibition

Table 4

Amino acid and NO_3^- levels in xylem exudates. Plant stems were cut just above the cotyledon, rinsed with distilled water, and the exudate collected for a 30 minute period at the end of the dark period (0 hr.) and after 6 hours in the light. Xylem sap samples were obtained from 11 to 22 days after incubation. Total α -amino nitrogen (Total Org N), and total α -amino and NO_3^- nitrogen (Total N) are listed below each set of samples

Plant age	mM					
	11		13		15	
	0 hr.	6 hr.	0 hr.	6 hr.	0 hr.	6 hr.
Asn	7.97	6.03	7.44	1.22	4.16	2.9
Asp	0.92	0.64	0.62	1.28	0.66	0.9
Gln	1.2	0.62	1.01	0.26	0.3	0.33
Glu	0.18	0.15	0.15	0.09	0.1	0.13
Hse	6.32	3.43	4.15	6.04	1.8	1.38
Ser	0.99	0.51	0.55	0.09	0.19	0.2
Ala	0.11	0.06	0.06	0.02	0.05	0.03
Arg	0.36	0.2	0.24	0.07	0.08	0.09
Thr	0.65	0.31	0.44	0.1	0.2	0.2
Tyr	0.17	0.06	0.1	0.02	0.03	0.03
His	0.62	0.24	0.36	0.07	0.14	0.13
Val	1.56	0.64	0.87	0.15	0.26	0.26
Phe	0.41	0.19	0.22	0.03	0.06	0.06
Ile	0.2	0.08	0.16	0.06	0.09	0.07
Leu	0.2	0.08	0.16	0.06	0.09	0.07
Lys	0.3	0.17	0.2	0.07	0.07	0.08
NO_3^-	2.0	1.2	1.99	5.8	11.2	7.8
Total Org N ^a	22.2	13.4	16.7	9.6	8.3	6.9
Total N ^a	24.2	14.6	18.7	15.4	19.5	14.7

a) sum of molarities of nitrogen containing compounds

continued...

Table 4 continued.
Amino acid and NO_3^- levels in xylem exudates

Plant age.	mM					
	18		20		22	
	0 hr	6 hr	0 hr	6 hr	0 hr	6 hr
Asn	2.63	1.69	2.1	2.83	2.47	1.97
Asp	1.18	1.1	1.41	0.3	0.61	0.87
Gln	0.36	0.33	0.33	0.5	0.18	0.23
Glu	0.17	0.11	0.17	0.07	0.12	0.11
Hse	1.23	0.96	1.28	1.46	1.67	1.67
Ser	0.18	0.13	0.13	0.16	0.08	0.11
Ala	0.06	0.03	0.04	0.01	0.04	0.03
Arg	0.09	0.07	0.08	0.1	0.04	0.12
Thr	0.15	0.11	0.14	0.17	0.11	0.12
Tyr	0.03	0.02	0.02	0.04	0.02	0.03
His	0.12	0.1	0.09	0.09	0.05	0.05
Val	0.24	0.19	0.021	0.24	0.13	0.15
Phe	0.05	0.04	0.04	0.06	0.02	0.02
Ile	0.11	0.09	0.09	0.06	0.05	0.07
Leu	0.1	0.07	0.08	0.07	0.08	0.11
Lys	0.09	0.08	0.08	0.17	0.07	0.11
NO_3^-	6.8	5.5	6.8	5.0	8.9	6.5
Total Org N ^a :	6.8	5.1	6.1	6.3	5.7	5.8
Total N ^a :	13.6	10.6	12.9	11.3	14.6	12.3

a) sum of molarities of nitrogen containing compounds

concentrations varied from 5 to 11 mM (figure 13c).

Cotyledons remained attached to plant stems throughout the 8 to 22 day period. During this time a qualitative change in the external appearance of the cotyledons was observed to take place. From 8 through to about 13 days cotyledons remained large (9 to 11 mm. diameter), while by 19 days after imbibition cotyledons were much reduced in volume (4 to 5 mm. diameter) and pronounced wrinkling was evident.

1.9 Asparaginase activity in detached leaves from soil grown plants

Feeding experiments, to investigate the affects of various compounds on asparaginase activity, were routinely performed using half expanded leaves. The procedure involved detaching leaves from soil grown plants at the end of the dark period, placing the petioles into treatment solutions and illuminating the leaves for a 2 to 3 hour period. An increase in asparaginase activity was observed when distilled water was fed to half expanded leaves for a three hour period in the light (table 5). An increase was also observed when several amino acids, NO_3^- , or NH_3 (all supplied at 8 mM, pH 6.3) were added to feeding solutions (table 5). But when a mixture of amino acids (6 mM asparagine, 6 mM glutamine, and 3 mM of both aspartate and glutamate) was fed to the leaves, no increase in activity resulted. The additions of glutamine, asparagine or NO_3^- resulted in the recovery of more asparaginase activity during the 3 hour feeding experiment than distilled water alone, and as a result, asparagine was routinely added to solutions during feeding experiments. The optimal concentration of asparagine for feeding experiments was determined to be 6 to 8 mM. The observed

Table 5

Asparaginase activity in detached, soil-grown leaves. Petioles of half expanded leaves were cut under water and placed into solutions at the end of the dark period. Samples were taken at this time (0 hour) and after 3 hours of treatment in the light, for determination of asparaginase activity. Concentrations of components in feeding solutions were 8 mM, except the mixed feeding solution contained: 6mM asparagine, 6mM glutamine, 3mM aspartate, 3mM glutamate. All solutions were at pH 6.3. The results were averaged from 3 separate experiments. Activities are expressed as a percentage of the value given by the asparagine fed treatment, because this treatment was used as a basis for comparison for subsequent feeding experiments. *in vivo* values are for the change in attached leaves during the same 3 hour period.

treatment	% activity $\pm$ SD (n=3) asparagine treatment	
0 hrs.	45	± 18
3 hrs.		
H ₂ O	84	± 14
Asn	100	-
Asp	64	± 11
Gln	115	± 15
Glu	86	± 18
NO ₃	113	± 12
NH ₄	88	-
Mixture	44	-
<i>in vivo</i>	106	-

increase in asparaginase activity during feeding experiments with added asparagine approximated the increase occurring in leaves attached to the plant during the same interval of time (*in vivo* activity, table 5). Based on the results from the experiments described below (section 1.10), only soil grown plants were used for feeding experiments.

1.10 Asparaginase activity in detached leaves from hydroponic plants

The increase in asparaginase activity observed in detached, half expanded leaves obtained from soil grown plants could not be demonstrated in leaves obtained from hydroponically grown plants (tables 7,8). During a 3 hour feeding experiment, a decrease in asparaginase activity was observed in detached hydroponic leaves fed water or asparagine (see tables 7 and 8). However as already noted (section 1.4) leaves that remained attached to hydroponically grown plants over the same period did show an increase in activity (see table 8). The decrease in asparaginase activity in detached leaves from hydroponic plants was not due to a change in fresh weight during the feeding experiment (i.e. water stressed leaves that would rapidly recover their water balance when placed into the feeding solutions) since the same trend was apparent when asparaginase activity was expressed on a protein basis (table 8). Analysis of xylem exudates from soil and hydroponically grown plants of the same age and obtained at the same times (at the end of the dark period and after 5 hours of light) were similar, the only notable differences were in the levels of asparagine and homoserine which were 2 fold higher in exudates from soil grown plants (table 6).

In an attempt to determine whether the contents of xylem sap were

Table 6

Analysis of xylem sap from soil and hydroponic plants. Shoots were removed from plants above the cotyledon (soil plants) or cotyledonary scar (hydroponic plants), root stumps rinsed with distilled water, and exudate collected for 30 minutes. Sap was collected at the end of the dark period (0 hr) and after 5 hours of light (5 hr). An estimate of the total nitrogen (based on 7 amino acids and NO_3^-) is listed below each column (total N)

amino acid concentration (mM)				
N	soil 0 hr	hydroponic 0 hr	soil 5 hr	hydroponic 5 hr
Asn	4.70	2.00	4.20	2.20
Asp	0.56	0.56	0.61	0.52
Gln	0.29	0.14	0.38	0.30
Glu	0.13	0.19	0.10	0.13
Hse	1.60	0.85	1.7	0.60
Ser	0.14	0.09	0.14	0.10
Arg	0.07	0.06	0.10	0.07
NO_3^-	9.2	11.8	7.2	14.2
Total N ^a	16.7	15.7	14.5	18.1

^a) sum of molarities of nitrogen containing compounds

responsible for the differences noted in the feeding experiments between detached soil and hydroponic leaves, 2 experiments were performed. The first involved feeding xylem sap collected from soil grown plants, before the end of the dark period, to half expanded leaves detached from hydroponic plants at the end of the dark period, for 3 hours in the light. The opposite experiment i.e. hydroponic sap fed to half expanded soil grown leaves was also performed. In the second experiment shoots from soil and hydroponically grown plants, detached above the cotyledon or cotyledonary scar, were placed into distilled water for a 24 hour period before feeding half expanded leaves 8 mM asparagine for 3 hours in the light.

When xylem sap obtained from soil grown plants was fed to detached leaves from hydroponic plants no increase in asparaginase activity was observed (table 7A). On the other hand, half expanded leaves from soil grown plants fed hydroponic xylem exudate demonstrated an increase in asparaginase activity, although the increase in activity was less than that observed in leaves fed 8 mM asparagine (table 7A). In this experiment wilting was noted in several of the xylem fed leaves after 2.5 hours of feeding, possibly due to the viscous consistency of the exudate. The experiment was therefore repeated using xylem exudate that was diluted 4-fold in distilled water (table 7B). After 3 hours of feeding 4-fold dilute xylem exudate to half expanded leaves, about 20% of the leaves had just started to become limp. Asparaginase activity increased in soil grown half expanded leaves fed either soil or hydroponic exudate diluted 4-fold, while hydroponic half expanded leaves fed soil or hydroponic exudate decreased in activity (table 7B).

In the second experiment, shoots of soil or hydroponic plants were

Table 7

Effect of feeding xylem sap on asparaginase activity in detached leaves. Xylem sap was collected from soil and hydroponic plants before the end of the dark period for 30 minutes. (A) At the end of the dark period soil xylem sap was fed to half expanded hydroponic leaves, and hydroponic xylem exudate fed to half expanded soil leaves for 3 hours in the light. Asparagine (8 mM, pH 6.3) was also fed to soil and hydroponic leaves. (B) Xylem exudates were diluted 4 fold prior to feeding as in (A). For both experiments asparaginase activity was determined at the end of the dark period (0 hr) and after 3 hours in the light (3 hr). Values are expressed % 0 hour asparaginase activity.

Treatment	% 0 hour activity
<u>A hydroponic plants</u>	
0 hr	100
3 hr	
+8 mM asn	33
+soil exudate ^a	22
<u>soil plants</u>	
0 hr	100
3 hr	
+8 mM asn	587
+hydroponic exudate ^a	317
<u>B hydroponic plants</u>	
0 hr	100
3 hr	
+8 mM asn	54
+soil exudate ^b	19
+hydroponic exudate ^b	38
<u>soil plants</u>	
0 hr	100
3 hr	
+8 mM asn	328
+hydroponic exudate ^b	156
+soil exudate ^b	297

a. xylem exudate was not diluted, wilting was noted after 2.5 hours.

b. xylem exudate diluted 4-fold, wilting just noted after 3 hours.

placed into distilled water for a 24 hour period in order to rinse the xylem free of constituents prior to feeding 8 mM asparagine for 3 hours in the light. A decrease in asparaginase activity was observed in half expanded hydroponic leaves during the feeding experiment, although the decrease was not as great as that observed in newly detached hydroponic leaves (table 8). Asparaginase activity in half expanded soil grown leaves flushed with water for 24 hours did not change during the feeding experiment, even though over the same time period newly detached leaves doubled their activity (table 8).

The only other observed difference, in relation to asparaginase activity between soil and hydroponically grown plants was in the apparent K_m for asparagine (no differences were observed in the V_{max} , see section III.1).

III Asparaginase activity: feeding experiments with MSO and AOA

Since asparaginase activity increases during the light period, a corresponding increase in the production of NH_3 may be detectable. To attempt to detect changes in the production of NH_3 it was necessary to inhibit the activity of glutamine synthetase, which normally maintains NH_3 at a low concentration (see diagram below), by using methionine sulfoximine (MSO). Furthermore, since photorespiratory nitrogen metabolism is responsible for high rates of NH_3 generation in the light, the transamination inhibitor aminooxyacetate (AOA) was added to arrest the production of NH_3 from photorespiration. Under these conditions, accumulation of NH_3 from asparaginase activity might be detectable.

Table 8

Asparaginase activity in detached hydroponic and soil grown leaves. (A) Half expanded leaves were removed from hydroponic plants at the end of the dark period, fed water or 8 mM asparagine (pH 6.3) for 3 hours in the light. Asparaginase activity was assayed at the end of the dark period (0 hr.) and after 3 hours of feeding (3 hr.). Asparaginase activity was also determined in half expanded leaves attached to plants over the 3 hour experiment (*in vivo*). (B) Shoots were cut above the cotyledons or cotyledonary scar and placed into distilled water for 24 hours (regular photoperiod) prior to detaching half expanded leaves at the end of the next dark period and feeding for 3 hours in the light (24 hr. H₂O). Asparaginase activity was determined at the end of the dark period (0 hr.) and after 3 hours of feeding (3 hr.). Asparaginase activities, obtained from half expanded soil grown leaves treated in the same manner as the hydroponic leaves, are listed for comparison. Asparaginase activities are expressed on a fresh weight and on a protein basis, and as % 0 hour activity.

Treatment	nmol asp/min g.f.r.wt.	% 0 hr.	nmol asp/min mg protein	% 0 hr.
<u>hydroponic plants</u>				
A 0 hr.	36.4	100	0.7	100
3 hr.				
+8 mM asn	12.2	34	0.27	39
<i>in vivo</i>	88.7	224	1.82	260
B 24 hrs. H ₂ O				
0 hr.	50.2	100	1.15	100
3 hr.				
+8 mM asn	39	78	1.04	90
<u>soil plants</u>				
A 0 hr.	17.9	100	0.41	100
3 hr.				
+8 mM asn	39.6	221	0.93	227
<i>in vivo</i>	70.4	393	1.39	339
B 24 hrs. H ₂ O				
0 hr.	40.5	100	1.11	100
3 hr.				
+8 mM asn	41.3	102	1.20	108

Experiments using MSO (Keys et.al. 1978, Kumar et.al. 1984, Berger and Fock 1983a,b, 1985, Platt and Anthon 1981, Platt and Rand 1982, Ikeda et.al. 1984, Achhireddy et.al. 1983) or MSO and AOA (Yamaya and Matsumoto 1985, Martin et.al. 1983) have been used to demonstrate that NH_3 levels, resulting from photorespiratory sources, increase in the light. The MSO/AOA feeding experiment was therefore performed to examine whether or not NH_3 production could result from asparaginase activity during the light. It was established that MSO (0.5 to 5 mM) or AOA (2 to 8 mM) had no inhibitory effect on asparaginase itself, when these compounds were added to crude enzyme extracts *in vitro*.

For these experiments both stage 3 and half expanded leaves were fed solutions of either water or 8 mM asparagine containing either 1 mM MSO, 4 mM AOA or both MSO (1 mM) and AOA (4 mM). Leaves were fed for 2.5 hours in the light starting either at the end of the dark period (0

hour) or after 14 hours of the light period. When stage 3 leaves were fed the samples included the next older leaf, whose stipules enclosed the stage 3 leaf and apex since leaves at this early stage do not actively transpire (Urquhart and Joy 1982). The levels of NH_3 in the leaf extracts were analysed by either microdiffusion or with a Beckman amino acid analyser.

The trends in NH_3 levels observed from MSO, AOA, or MSO+AOA feeding treatments were similar for leaves treated with or without addition of asparagine, in both stage 3 and half expanded leaves, although levels of NH_3 in stage 3 leaves were lower than those observed in half expanded tissues (table 9). Leaves (dark or light treated) displayed increased NH_3 levels when supplied with MSO, compared with control leaves fed only water or asparagine (ranging from 1.5 to 3.5 fold in stage 3 leaves and 5 to 14.5 fold in half expanded tissues). This result is consistent with the role of glutamine synthetase in the assimilation of NH_3 in leaf tissues. The addition of AOA alone to feeding solutions did not noticeably alter levels of NH_3 within leaves, yet when AOA was added together with MSO the high values of NH_3 observed with MSO were reduced and approximated those of the control treatments, suggesting that an AOA sensitive reaction (presumably photorespiration) is responsible for production of most of the NH_3 in the leaf.

An increase in NH_3 levels during the light period in the presence of MSO and AOA, which might have resulted from increased asparaginase activity, was not observed. This experiment suggests a) that

Table 9

Ammonia accumulation in leaves and effects of inhibitors. Leaves were detached at end-of dark (0hr.) and light periods (14 hr.) then supplied with water or asparagine (8 mM) for 2.5 hours in the light, after which the levels of NH_3 were determined. Concentrations of MSO and AOA were 1 mM, and 4 mM respectively (pH 6.3). Stage 3 leaves were fed while still attached to the next older leaf

Leaf age: Treatment	NH_3 levels ($\mu\text{mol/gm.fr wt.}$)			
	stage.3		half expanded	
	0 hr.	14 hr.	0 hr.	14 hr.
H_2O	0.79	0.89	1.61	1.29
$\text{H}_2\text{O}+\text{MSO}$	2.18	2.25	8.44	8.60
$\text{H}_2\text{O}+\text{AOA}$	-	-	1.04	0.91
$\text{H}_2\text{O}+\text{MSO}+\text{AOA}$	0.79	0.82	1.78	1.98
Asn	0.78	1.53	0.97	0.83
Asn+MSO	3.11	3.35	14.09	10.70
Asn+AOA	-	-	1.33	0.75
Asn+MSO+AOA	0.92	0.89	1.76	1.60

transamination-dependant NH_3 sources greatly exceed sources of NH_3 that are not dependant upon transamination (including asparaginase) and b) that the increase in extractable asparaginase activity during the light period observed *in vitro* may not reflect a functional increase *in vivo*. To ensure that MSO was inhibiting glutamine synthetase during the feeding experiments, MSO was fed for 2.5 hours in the light to half expanded leaves at the end of the dark period. Extractable glutamine synthetase activity was reduced by over 90% in leaves fed 0.5 mM MSO, the lowest concentration fed (figure 14). The levels of NH_3 were analysed in the extracts from tissues supplied with MSO, and an 8-9 fold increase in the levels of NH_3 was observed (figure 14).

The possibility that asparaginase activity might be inhibited by either MSO or AOA had already been checked (see above) and no adverse effects on asparaginase activity (*in vitro*) were found. However, asparaginase activity was also assayed in leaf extracts obtained from the MSO feeding experiment (figure 14), and a decrease in activity below that of the 0 hour control resulted, in the presence of MSO. Over the 2.5 hour feeding period in the light, increased asparaginase activity was observed in the control treatment (8 mM asparagine). One explanation for the *in vivo* inhibition of asparaginase by MSO could be that the increased NH_3 levels may inhibit the production of asparaginase in the light. However, asparaginase activity increased in half expanded leaves (with active glutamine synthetase activity) when 8 mM NH_3 was fed to detached leaves for a 3 hour period in the light (see table 5).

To determine if MSO had an effect on levels of established asparaginase activity in the light, MSO and asparagine, or asparagine

Figure 14

Effects of methionine sulfoximine on asparaginase activity, glutamine synthetase activity, and ammonia levels in detached leaves. Half expanded leaves were placed into solutions containing 8 mM asparagine with varying levels of methionine sulfoximine (MSO), at the end of the dark period (initial activities and NH_3 levels, solid symbols), and fed for 2.5 hours in the light (open symbols). Enzyme activities are expressed as a percent of control (no MSO) activity: asparaginase (o), glutamine synthetase (Δ), ammonia levels ($\square$)

alone were fed to half expanded leaves for 2 hours, 6 hours after the onset of the light period. Enzyme extracts were prepared from leaves after 6 hours of light, as well as after 1 or 2 hours of feeding, and assayed for asparaginase activity (table 10). Extracted asparaginase activity decreased over the 2 hour feeding experiment in the presence or absence of MSO in the feeding solution. The decrease in activity was not due to a change in fresh weight during the feeding experiment (if the leaves had been water stressed prior to the feeding experiment they may have increased in fresh weight when placed in feeding solutions) since the decrease was also observed when activities were compared on a protein basis (table 10). The decrease in asparaginase activity was different from the decrease in activity noted at the end of the dark period (figure 11). In the above feeding experiment activity decreased immediately, during the first hour, while for intact plants reduction of activity at the end of the photoperiod began only after a one hour lag (figure 11).

The decrease in asparaginase activity in detached leaves fed MSO during the light period (table 10) was not as pronounced as the decrease in activity observed in detached leaves fed MSO at the end of the dark period (figure 14). A possible explanation for this observation is that asparaginase activity, once established in the light, requires about 4 to 6 hours of dark for a marked decrease in activity to take place (figure 11), while asparaginase at the end of the dark period continues to decrease if the dark period is extended (see section II.1, figure 15).

The results obtained from the experiments conducted at the end of the dark period (figure 14) or after 6 hours in the light (table 10), point to two possible reasons why changes in NH_3 levels were not observed in

Table 10

Effects of MSO or asparagine on asparaginase activity in detached leaves during the light period. Half expanded leaves were placed into solutions containing 8 mM asparagine or 8 mM asparagine and 1 mM MSO (at pH 6.3) after 6 hours in the light period (initial activity), and fed for 2 hours in the light. Asparaginase activity is presented on a per fresh weight (two experiments) or mg protein basis. Values are expressed as % initial asparaginase activity

treatment	% initial activity		
	per gm. fr. wt.		per mg protein
initial activity	100	100	100
1 hour ASN	86	79	77
+MSO	77	nd ^a	85
2 hour ASN	60	55	61
+MSO	52	nd ^a	56

a) not determined

vivo, during the MSO/AOA feeding experiment. The first reason arises from the observation that increased asparaginase activities during the first 2-3 hours of the light period in control treatments of feeding experiments did not take place when MSO was added to the feeding solutions. It is possible that increased NH_3 levels within leaf tissues, due to MSO, may have a negative effect upon the increase of asparaginase activity. The second reason relies on the observation that within the light period asparaginase activity decreased over the course of a 2 hour feeding experiment in the presence or absence of MSO. This latter experiment demonstrates that an increase in NH_3 levels, resulting from increased asparaginase activity in the light, can not be demonstrated by using feeding experiments.

II.1 Light and asparaginase activity

Three groups of experiments were conducted to investigate whether or not the observed increase in asparaginase activity was a response to light. The initial experiments considered the possibility that diurnal variation in asparaginase activity might be an entrained circadian rhythm. In the second set of experiments a dose-response relationship between energy fluence rate and asparaginase activity was investigated, and the final series of experiments examined the participation of the light reactions in the increase of asparaginase activity. All of these experiments were carried out using soil grown plants.

II.1 Asparaginase activity and extended dark/light periods

Plants were subjected to extended periods of light or dark and asparaginase activity was determined in young leaf tissue (figure 15). A prolonged light period resulted in the maintenance of high levels of activity. When the dark period was extended, the levels of extractable activity continued to decrease slowly to a low value (figure 15). The decrease in activity in the dark also occurred when the temperature was held at 25°C (rather than dropping to 18°C as in the regular photoperiod). These observations indicated that the diurnal variation in asparaginase activity was not an entrained circadian rhythm nor was it due to temperature alterations during the photoperiod. Furthermore, they indicated that light may be involved in the increase of asparaginase activity.

II.2 Asparaginase activity and energy fluence rates

Figure 15

Effects of prolonged light or dark periods on asparaginase activity in young leaves. Young (stage 3) leaves were obtained from soil grown plants that were maintained in continued light (○) or dark (●) periods. Diurnal variation of asparaginase (Δ) during a regular photoperiod (16 hours of light, 23°C, and 8 hours of dark 18°C) is presented for comparison. Asparaginase activity decreased in dark-treated plants (●) at 18°C, or 25°C. Asparaginase activity is expressed as nmol asp/min/gm fr. wt.

Plants were placed in black plexiglass boxes which helped minimize any stray light during the experimental period and which also provided support for neutral density filters (layers of cheesecloth and miracloth) that were used to vary the energy fluence rates from 1 to 10 W/m² (one group of plants were left in the dark over the experimental period, 0 W/m² in figure 16). Before the onset of the light period, neutral density filters were placed 20 cm above the plants. Half expanded leaves were harvested and assayed for asparaginase activity at the end of the dark period (0 hour) and after 4 or 6 hours of exposure to varying energy fluence rates (figure 16a). Energy fluence rates (measured as photosynthetically active radiation, PAR) were obtained at the end of each experiment. As the energy fluence rate increased, a corresponding increase was observed in accumulated asparaginase activity for both the 4 and 6 hour treatment periods. Since the trends in both experiments were similar, asparaginase activities determined for the dark treatments were subtracted from the activities of the other treatments, and the data pooled (figure 16b, $r = 0.91$). From extrapolation of the line (figure 16b) it appeared that no increase in asparaginase activity occurred below an energy fluence rate of approximately 0.8 W/m².

A time course of asparaginase activity over the first 6 hours of the light period was carried out at 2 energy fluence rates (4 and 8.3 W/m²) using half expanded leaves (figure 17). During the first hour of the light period decreased activity was observed for both energy fluence rate treatments, and this is consistent with earlier observations on half expanded leaves (figure 11). The decrease in asparaginase activity during the first hour in the 4 W/m² treated plants was more pronounced than the decrease observed in the 8.3 W/m² treatment. After the second hour

Figure 16

Effect of energy fluence rate on asparaginase activity (a) Plants were exposed to varying energy fluence rates for 4 (●) or 6 (▲) hours after which time asparaginase activity (nmol asp/min/gm fr wt) was assayed in half expanded leaves. The dark treatments were subtracted from the activities in (A) and the data pooled in (B) ($r=0.91$)

Figure 17

Time course of asparaginase activity at two different energy fluence rates. Soil grown plants were exposed to energy fluence rates of 40 W/m² (Δ) or 8.3 W/m² (o) and asparaginase activity (nmol asp/min/gm fr wt.) in half expanded leaves was assayed during a 6 hour period. Values represent mean $\pm$ SD (n=3).

in the light period, asparaginase activity obtained from both treatments increased, although the levels observed in the 4 W/m² treated plants were much lower than those obtained from the 8.3 W/m² treatment.

II.3 Asparaginase activity and photosynthetic inhibitors

To examine the relationship between light and asparaginase activity in more detail, photosynthetic inhibitors were fed to half expanded leaves, and effects on asparaginase activity as well as on photosynthetic activity were observed. Photosynthetic activity was initially assayed in crude chloroplast membrane preparations by measuring the rate of DCPIP reduction, an electron acceptor that changes colour as it becomes reduced. Using the method of DCPIP reduction, the herbicides DCMU and atrazine were observed to inhibit *in vitro* photosynthetic activity in chloroplast preparations at low concentrations (figure 18, 90% inhibition of photosynthesis with 1 μ M DCMU, or 3 μ M atrazine). These inhibitors had no effect upon *in vitro* asparaginase activity up to 10 μ M DCMU or atrazine concentrations, the highest concentrations assayed.

A range of concentrations (0 to 100 μ M) of DCMU or atrazine were fed to half expanded leaves for 2.5 hours in the light. Extracts were obtained at the end of the dark period (0 hour) and after the 2.5 hour feeding in the light, and assayed for asparaginase activity as well as photosynthetic activity. For these experiments *in vivo* photosynthetic activity was measured either by assaying O₂ evolution from leaf pieces (figure 20) or measuring *in vivo* chlorophyll *a* fluorescence (figures 19, 20, 22). These methods were employed since it was observed that *in vitro* measurements of photosynthesis (DCPIP reduction and O₂ evolution) using

Figure 18

Effect of DCMU and atrazine on *in vitro* photosynthetic activity.

Chloroplast membranes were prepared from half expanded leaves and photosynthetic activity (estimated by DCPIP reduction) was determined in the presence of varying concentrations of DCMU (●) or atrazine (■). Values are presented as a percentage of the control (no inhibitor) treatment.

chloroplast preparations obtained from herbicide feeding experiments yielded variable results possibly due to reversible herbicide binding (see appendix 2). The measurement of *in vivo* chlorophyll *a* fluorescence was more convenient than estimation of O_2 evolution from leaf pieces and this latter method was used only to confirm the observations obtained from *in vivo* chlorophyll *a* fluorescence.

The fluorescence induction curve (F_{685}) was found to change in leaves fed with increasing DCMU or atrazine concentrations (see Baker and Bradbury 1981). The initial 'O'-'P' transients were not altered by increasing DCMU treatments (figure 19, 20; $\Delta O-P$); although a change was observed in the 'P'-'T' portion of the induction curve (figures 19, 20, 22; $\Delta P-T$). Similar results were noted in atrazine fed leaves except that the decrease in $\Delta P-T$ was more pronounced with increasing atrazine concentrations (figure 19, 22). The decrease in $\Delta P-T$ noted in leaves fed increasing concentrations of DCMU approximated the decrease observed in O_2 evolution from leaf pieces (figure 20).

During the 2.5 hour light period asparaginase activity increased in control treatments (figures 21, 22). When 10 μM or more of DCMU or atrazine were fed to half expanded leaves, no increase in asparaginase activity was observed. The response of asparaginase activity to increasing inhibitor concentrations in the feeding experiments was similar for either the DCMU or atrazine treatments (see figure 21) even though the decrease in photosynthetic activity differed in both herbicide treatments. Photosynthetic activity decreased in the order of 10% (DCMU) or 80% (atrazine) in leaves fed 10 μM herbicide and at this concentration of herbicide only a marginal increase in asparaginase

Figure 19

Effect of DCMU or atrazine on the fluorescence induction curve. Half expanded leaves, obtained at the end of the dark period, were fed 0 (A), 20 (B), 50 (C), or 100 (D) μM of DCMU or atrazine for 2.5 hours in the light (energy fluence rate 10 W/m^2 , PAR). After this time, photosynthetic activity was estimated using *in vivo* chlorophyll *a* fluorescence. The initial rise in fluorescence along the induction curve, 'O-P', arises from reduction of the plastoquinone (PQ) pool, while transient increases in fluorescence between 'P' and 'T' result from electron flow from the PQ pool to photosystem I. Inhibitors such as DCMU or atrazine block electron flow from the primary electron acceptor Q to PQ, which results in continued fluorescence after 'P' on the fluorescence induction curve (for details see Baker and Bradbury 1981). The changes in 'P-T' transients ($\Delta \text{P-T}$), with increasing inhibitor concentration, are plotted in figures 20 and 22.

Figure 20

Effect of DCMU on O_2 evolution from leaf pieces and *in vivo* chlorophyll *a* fluorescence. Half expanded leaves were detached at the end of the dark period and fed varying concentrations of DCMU for 25 hours in the light (energy fluence rate 10 W/m^2 , PAR). After this time, photosynthetic activity was estimated using O_2 evolution from leaf pieces (Δ), or *in vivo* chlorophyll *a* fluorescence (Δ 'P-T' transients $\bullet$). The change in 'O-P' (Δ 'O-P' transients, $\circ$) are presented for comparison. Values for *in vivo* chlorophyll *a* fluorescence are expressed as a percent of the control (no DCMU) treatment. In all cases values represent mean $\pm$ SD (n=5).

Figure 21

Effect of DCMU or atrazine on asparaginase activity. Half expanded leaves were detached at the end of the dark period (initial asparaginase activity, solid symbols) and fed varying concentrations of DCMU (o) or atrazine (Δ) for 2.5 hours in the light (open symbols) (energy fluence rate 10 W/m^2 , PAR). After this time, asparaginase activity (nmol asp/min/mg fr. wt.) was assayed. Values, for the DCMU treatment only, represent mean $\pm$ SD (n=3).

Figure 22

Effect of DCMU or atrazine on asparaginase and photosynthetic activities. Half expanded leaves were detached at the end of the dark period (initial activities of asparaginase: solid symbols) and fed varying concentrations of DCMU or atrazine for 2.5 hours in the light (open symbols) (energy fluence rate 10 W/m^2 , PAR). Photosynthetic activity was estimated by *in vivo* chlorophyll *a* fluorescence ($\Delta'P-T'$ transients $\bullet$) and asparaginase activity (Δ). Values are expressed as a percent of the control treatment and represent mean $\pm$ SD (for asparaginase $n=3$, for $\Delta'P-T'$ transients $n=5$)

activity was observed. At higher herbicide concentrations asparaginase activity decreased below the levels noted at the onset of the light period.

To determine whether products from the light reactions required for the increase in asparaginase activity could be replaced by a final product of the dark reactions, DCMU was fed to half expanded leaves along with sucrose. Enzyme extracts were obtained at the end of the dark period (0 hour) and after 3 hours of feeding in the light. In the control treatment asparaginase activity increased during the feeding period (table 11). When 30 μ M DCMU was added to the feeding solution a slight increase in activity was noted over the 3 hour period. Asparaginase activity increased in leaves fed either 10 or 20 mM sucrose, compared with the control treatment, although when DCMU was added along with either 10 or 20 mM sucrose only a slight increase over the DCMU treatment was observed (table 11). These results suggest that a product of photosynthesis, not replaced by carbohydrate produced by the dark reaction, is required for the increase observed in asparaginase activity.

Table 11

Effect of DCMU and sucrose on asparaginase activity in detached leaves. Half expanded leaves were fed for 3 hours in the light either 30 μ M DCMU, 10 or 20 mM sucrose, or 30 μ M DCMU along with 10 or 20 mM sucrose. All solutions contained 8 mM asparagine and were at pH 6.3. Samples were obtained at the end of the dark period (0 hour) and after 3 hours in the light. Values are expressed as % activity of the asparagine only treatment

treatment	% activity asparagine treatment
0 hour	42
asparagine only	100
+ 30 μ M DCMU	56
+ 10 mM sucrose	117
+ 20 mM sucrose	126
+ 30 μ M DCMU + 10 mM sucrose	62
+ 30 μ M DCMU + 20 mM sucrose	61

III Studies on the mechanism of the light-dependent increase in asparaginase activity

When leaves are illuminated, a range of compounds change in concentration within the cell. Compounds such as reduced ferredoxin, ATP, and NADPH are products of photosynthesis while others including sugar phosphates, ADP, NADH, and some ions change in concentration due to pathways that are activated (or deactivated) in the light. Several mechanisms controlling enzyme activity are activated by light either as a result of photosynthesis, or by light absorbing pigments other than chlorophyll. These mechanisms include a) reduction of disulfide groups on enzymes by thioredoxin or light effect mediator (LEM), or b) light induced alterations in membrane permeability mediated by the reversible photochromic pigment phytochrome, or c) activation of enzymes by the Ca^{2+} -binding protein calmodulin. In order to understand the mechanism underlying the increase in asparaginase activity in the light, experiments were performed to examine whether products of the light reactions, or mechanisms dependent upon light, affected asparaginase activity. The involvement of light reaction products, the participation of phytochrome or calmodulin, and the role of protein synthesis in the light dependent increase of asparaginase activity were investigated.

III.1 Is there a change in enzyme kinetics associated with increased asparaginase activity?

Extracts from half expanded leaves, obtained at the end of the dark period and after 6 hours in the light, were desalted (Tris HCl KCl DTT buffer) and assayed over a range of asparagine concentrations.

Hyperbolic kinetics were observed for asparaginase (for asparagine) extracted at the end of the dark period or in the light. A decrease in the K_m for asparagine and an increase in the V_{max} took place in the light (figure 23, 24) although it was observed that the K_m values were variable. In half expanded leaves from soil-grown plants the K_m for asparagine at the end of the dark period ranged from 10 to 16 mM (average K_m 11.1 ± 2.3 mM, $n=3$) and in the light from 4 to 8 mM (average K_m 7.1 ± 3.1 mM, $n=3$). The K_m for asparagine in half expanded leaves from hydroponic plants also decreased during the light period. At the end of the dark period the K_m ranged from 14 to 18 mM, 15.8 ± 3 mM ($n=4$), and in the light the K_m was 7.2 ± 0.2 mM ($n=3$). The V_{max} from either soil or hydroponically grown plants increased 2-fold in the light from approximately 120 nmol/min/g.f.r.wt. at the end of the dark period to approximately 200 nmol/min/g.f.r.wt. in the light. These results indicate that the affinity of asparaginase for asparagine changes in the light; the kinetic change was more pronounced in asparaginase obtained from hydroponic plants (for other differences in asparaginase activity between hydroponic and soil grown plants see section I.10).

III.2 Asparaginase activity and products of the light reactions

Electron flow through photosystems II and I in the light leads to the synthesis of ATP and the reduction of ferredoxin, NADP (and NAD via NAD kinase), and thioredoxin or LEM. These compounds were examined directly or indirectly, using incubation experiments (see section I.3), to see if they played a role in increasing asparaginase activity in crude

Figure 23

Michaelis-Menten kinetics of asparaginase. Half expanded leaves were obtained from soil grown plants at the end of the dark period (●), or after 6 hours in the light (○) and assayed for asparaginase activity (nmol asp/min/gm fr wt) in varying concentrations of asparagine. The Lineweaver-Burk plot is presented in figure 24

Figure 24

Michaelis-Menten kinetics of asparaginase: Lineweaver-Burk plot. Half expanded leaves were harvested at the end of the dark period (●) or after 6 hours in the light (○) and assayed in increasing concentrations of asparagine (see figure 23). Values for K_m and V_{max} were estimated by linear regression.

K_m (asn) - at end of the dark period: 15.9 mM

- after 6 hours in the light: 7.1 mM

V_{max}

- at end of the dark period: 4.4 nmol asp/min/mg protein

- after 6 hours in the light: 8.0 nmol asp/min/mg protein

preparations. Half expanded leaf material was harvested at the end of the dark period or after 8 hours of light for use in incubation experiments.

Asparaginase could theoretically respond to an added compound during incubation in 4 ways:

1) compounds could have a stimulating effect on asparaginase activity during incubation, and therefore asparaginase activity after the 30 minute incubation period would be greater than initial activity, i.e. $\geq 100\%$.

2) additions could stabilize activity (i.e. increase asparaginase activity above the 4 mM asparagine treatment but not above initial activity; 70-99% of initial activity).

3) additions could have no effect on asparaginase activity and therefore the activity would approximate the 4 mM asparaginase treatment (60-69% of initial activity).

4) compounds could reduce activity below that of the 4 mM asparagine control treatment, but not below the treatment without asparagine (activity 40-59%), or activity could be inhibited and decrease below that of the treatment without asparagine (activity $\leq 39\%$).

After incubation for 30 minutes, asparaginase obtained at the end of the dark period was observed to respond to the additions in 3 of the 4 possible ways (table 12). First, the compounds NADPH, NADH, reduced ferredoxin, K_2HPO_4 , and $Na_2S_2O_4 / NaHCO_3$ stabilized asparaginase during incubation (in the presence of 4 mM asparagine). Secondly, no effect on asparaginase activity was noted when GSSG, GSH, or DTT were added to asparaginase preparations (DTT marginally stabilized asparaginase activity). Thirdly, the addition of oxidized ferredoxin, MgADP, or MgATP

Table 12

Effect of photosynthetic products on asparaginase activity. Half expanded leaf material (obtained at the end of the dark period) was ground, centrifuged and desalted in Tris KCl CaCl_2 buffer prior to incubation for 30 minutes with 4 mM asparagine and the compounds of interest. Asparaginase activity was assayed at the start (0 min.) and end of preincubation. Activities are expressed as a % of the initial (0 min.) value.

Treatment (additions in mM)		% initial activity	
0 min.		100	100
30 min. preinc. plus.			
Asparagine	4	66	65
no additions		45	43
30 min. preinc. + 4 mM asn:			
red ferredoxin ^a	0.1 mg/ml	94	95
ox ferredoxin	0.1 mg/ml	34	32
$\text{Na}_2\text{S}_2\text{O}_4/\text{NaHCO}_3$	1.8 mg/ml	72	-
DTT	2.0	64	66
	10.0	66	66
NADPH	2.5	96	98
	2.0	90	-
	1.0	79	-
	0.5	77	-
	0.25	60	-
NADH	2.5	96	91
	1.0	82	-
GSSG	5.0	67	-
GSH	5.0	64	-
MgATP	5.0	5	6
	1.0	4	6
MgADP	5.0	8	6
	2.5	20	-
K_2HPO_4	10.0	88	92
	5.0	89	-
	2.5	74	-

a) ferredoxin was reduced with $\text{Na}_2\text{S}_2\text{O}_4/\text{NaHCO}_3$ (25 mg/ml $\text{Na}_2\text{S}_2\text{O}_4$ in 0.3 M NaHCO_3 , Wallsgrave et al. 1982) at the start of the preincubation.

resulted in a decrease in asparaginase activity below that observed in the treatment without asparagine. During the preincubation experiment no stimulation of asparaginase activity was observed. When asparaginase, harvested after 8 hours in the light period, was examined in the incubation experiments, results similar to those noted above for asparaginase obtained at the end of the dark period were observed.

The compounds that stabilized asparaginase in the above incubation experiment were added to asparaginase preparations during a 30 minute incubation period without substrate to see if asparagine could have affected the response of asparaginase to the additions (table 13). Addition of DTT, NADH, or NADPH did not stabilize asparaginase during incubation. The decrease of asparaginase activity in the presence of DTT, NADPH or NADH paralleled the decrease in activity in the control treatment without asparagine (table 13). The compounds K_2HPO_4 and glycerol were observed to stabilize asparaginase activity during incubation in the absence of asparagine. No activation of asparaginase was observed in the above experiments during preincubation. DTT (10 or 50 mM) was also added to asparaginase preparations and incubated for up to 2 hours ($\pm$ 4 mM asparagine, $34^\circ C$) and no change in asparaginase activity was observed compared with the control treatments.

NADPH (0.5 and 2.0 mM), reduced and oxidized ferredoxin (0.1 mg/ml final concentration), DTT (2 to 50 mM), or K_2HPO_4 (5 mM) were added to asparaginase assays (without any incubation) and no change in asparaginase activity was observed. The addition of MgATP to asparaginase assays, without incubation, decreased activity. Kinetic analysis indicated that MgATP inhibits asparaginase activity

Table 13

Effect incubating asparaginase with compounds of interest but without asparagine Half expanded leaf material was harvested in Tris KCl CaCl₂ buffer at the end of the dark period prior to incubation for 30 minutes with compounds of interest; no asparagine was added during incubation. Asparaginase activity was determined at the start and end of incubation. Values are expressed as a % of initial asparaginase activity (similar results were noted with preparations obtained during the light period).

Treatment (additions in mM)		% initial activity
0 minute		100
30 min. preincubation:		
no addition		45
30 min. preinc.(no asn).		
DTT	2.0	49
	10.0	51
NADPH	2.5	39
NADH	4.0	30
K ₂ HPO ₄	10.0	68
glycerol	20%	81
Casein	1%	50
EDTA	1 & 5	45

non-competatively (figure 25, 26). MgATP can bind asparaginase in the presence of asparagine ($K_{1/2}$ 0.24 mM for MgATP, i.e. $K_{1/2}$ enzyme+substrate figure 26B). In the absence of asparagine MgATP can bind to asparaginase at higher concentrations ($K_{1/2}$ 0.82 mM for MgATP, figure 26C).

The above results indicate that reduced products of the light reactions, (NADPH, and reduced ferredoxin, as well as NADH) help stabilize asparaginase harvested at the end of the dark or during the light periods but only in the presence of substrate.

To determine whether NADPH and reduced ferredoxin help stabilize asparaginase activity directly, or indirectly through ferredoxin-thioredoxin reductase or LEM, crude asparaginase preparations were partially purified prior to incubation with NADPH and reduced ferredoxin. Homogenates from half expanded leaves were passed through an S300 gel filtration column (fractionation range: Mr's 500-250000) to remove proteins such as LEM or ferredoxin-thioredoxin reductase (Mr's 30000-50000) from the asparaginase (Mr 68000, see figure 36) fraction. S300-Asparaginase was incubated for 30 minutes in 4 mM asparagine, along with compounds of interest prior to assaying asparaginase activity (table 14). Asparaginase activities in the 3 control treatments (i.e. initial activity, +4 mM asparagine, and no asparagine) approximated those observed earlier in crude enzyme incubations. The activities assayed after NADPH, reduced or oxidized ferredoxin, or $\text{Na}_2\text{S}_2\text{O}_4/\text{NaHCO}_3$ were added to S300 asparaginase preparations during incubation differed from that observed in crude preparations. Incubation with NADPH, reduced or oxidized ferredoxin resulted in a decrease in asparaginase

Figure 25

Inhibition of asparaginase activity by MgATP. Half expanded leaves were obtained after 6 hours in the light and assayed for asparaginase activity in varying concentrations of asparagine and 0.0 (○), 0.4 (□), 0.8 (▽), 1.2 (◇) mM MgATP. See figure 26 for Lineweaver-Burk plot and inhibitor constants.

Figure 26

Inhibition of asparaginase by MgATP: Lineweaver-Burk plot. Half expanded leaves were obtained after 6 hours in the light and assayed for asparaginase activity in varying concentrations of asparagine and 0.0 (○), 0.4 (□), 0.8 (▽), and 1.2 (◇) mM MgATP. (A) Lineweaver-Burk plot displaying mixed, non-competitive inhibition (i.e. V_{max} decreased, and K_m increased, see Engle 1981). The K_i values of MgATP inhibition were determined from secondary replots of the slopes (B) or intercepts (C) of the Lineweaver-Burk plot for various inhibitor concentrations. MgATP may bind the enzyme-substrate complex K_{ies} 0.24 mM (B), or bind the enzyme without substrate K_{ie} of 0.82 mM (C).

Table 14

Incubation of S300-asparaginase with products of the light reaction. Half expanded leaf material was ground, centrifuged and passed through an S300 column (all in Tris KCl CaCl_2 buffer) prior to incubation for 30 minutes with compounds of interest in the presence of 4 mM asparagine. Asparaginase activity was determined at the start and end of incubation. Values are presented as % initial activity.

treatment (additions in mM)		% initial activity
0 minute		100
30 min. preincubation:		
asparagine	4.0	65
no additions		41
30 min. preinc. + 4mM asn.		
red. ferredoxin	(0.1 mg/ml)	55
ox. ferredoxin	(0.1 mg/ml)	42
$\text{Na}_2\text{S}_2\text{O}_4/\text{NaHCO}_3$	(1.8 mg/ml)	72
NADPH	1.0	55
MgADP	5.0	6
	1.0	5
MgATP	5.0	.7
	1.0	6

activity which paralleled that of the 4 mM asparagine treatment, while $\text{Na}_2\text{S}_2\text{O}_4/\text{NaHCO}_3$ marginally stabilized activity. The additions of MgADP, or MgATP reduced asparaginase activity to the levels observed in crude asparaginase preparations. These results suggest that a compound of low or high molecular weight may be responsible for the stability of asparaginase activity *in vitro* in conjunction with a product of the light reactions.

Activation and deactivation of several light modulated enzymes is mediated by thioredoxin or LEM *in vivo* as well as *in vitro*, and in the absence of these proteins alterations in enzyme activity do not take place. If the decrease in asparaginase activity during incubation in crude preparations was mediated by thioredoxin or LEM, then no change in asparaginase activity after partial purification (S300) should be observed when LEM or thioredoxin have been separated from asparaginase. Since a decrease in activity was observed in the treatment without asparagine (table 14), the possibility exists that the S300-asparaginase preparation was contaminated with low (or high) molecular weight compounds that could affect activity. To examine this possibility, the first asparaginase fractions collected from the S300 column, with higher molecular weight contaminants, were treated separately from later eluting asparaginase fractions, contaminated with lower molecular weight compounds. Both of these asparaginase fractions were incubated for 30 minutes without any additions and asparaginase activity was determined before and after preincubation. S300-asparaginase from either pooled fraction decreased during incubation, and, in both cases, at the end of the incubation period, 40% of the initial asparaginase activity remained. This result indicates that the

decrease in asparaginase activity during incubation was not mediated by proteins such as ferredoxin-thioredoxin reductase or LEM.

In summary, the results from this group of experiments indicate that the compounds NADPH, NADH and reduced ferredoxin stabilize asparaginase in crude (G25 desalted) enzyme preparations in the presence of asparagine, although no activation of asparaginase was observed with any of these compounds. The protection of asparaginase activity in crude preparations by NADPH and reduced ferredoxin is controlled by a compound of lower, or higher molecular weight which is removed from asparaginase by S300 chromatography. However, this compound does not appear to mediate the decrease of asparaginase activity.

III.3 Asparaginase activity, pH and Mg^{2+}

As a result of electron flow through the photosystems, protons are shuttled into the lumen of the thylakoids while at the same time Mg^{2+} ions leave the thylakoids and enter the stroma. Due to the translocation of protons out of the stroma during photosynthesis, the stromal pH becomes more alkaline, changing by 1 pH unit (Werdan et.al. 1975). Changes in stromal pH as well as in Mg^{2+} concentration play a role in the regulation of several chloroplastic enzymes (Portis and Heldt 1976, Buchannan 1980). Asparaginase has been reported to be a cytosolic enzyme (Ireland and Joy 1983b). However, in view of the relationship between asparaginase and photosynthesis, the effects of pH and Mg^{2+} changes (responsible for regulation of other light-modulated enzymes) were investigated in crude asparaginase preparations.

Half expanded leaves were harvested at the end of the dark period or

after 8 hours in the light and assayed for asparaginase activity over a range of pH's. The physiological change in pH resulting from photosynthetic electron flow that is important in enzyme regulation spans pH 7 to 8 and therefore a pH range of 6 to 9 was investigated. Asparaginase preparations, obtained from the end of the dark or during the light periods, responded to increasing pH in similar manner (figure 27). Below pH 6.5, no activity was observed in either asparaginase preparation. Asparaginase activity increased with increasing pH up to pH 8.0, and remained active through to pH 9.0, the highest pH tested. No differences were observed between asparaginase preparations obtained at the end of the dark or during the light periods over the pH range of 7 to 8.

Asparaginase preparations (desalted in Tris HCl, $\pm$ KCl) harvested at the end of the dark period or after 5 hours in the light were assayed with a range of Mg^{2+} ion concentrations (0 to 10 mM). With increasing Mg^{2+} ion concentration no change in asparaginase activity was noted if the enzyme was desalted in the presence of KCl (figure 28). When asparaginase was desalted in the absence of KCl, Mg^{2+} was observed to inhibit asparaginase activity in light and dark preparations (figure 28). No stimulation of asparaginase activity was observed with Mg^{2+} . Asparaginase (desalted with Tris HCl + KCl) was also incubated with 3 mM Mg^{2+} in the presence or absence of asparagine for 30 minutes prior to assaying asparaginase activity and no change in activity was observed in the Mg^{2+} treatments compared with the control treatments ($\pm$ 4 mM asparagine). The addition of 2 mM EDTA during extraction, assay, or incubation had no effect on asparaginase activity (enzyme desalted with Tris HCl + KCl).

Figure 27

Asparaginase activity and pH. Half expanded leaves were harvested at the end of the dark period (B) or after 6 hours in the light (A) and assayed for asparaginase activity (nmol asp/min/gm fr wt) from pH 5.6 to 9.0. Buffers were all at a concentration of 200mM and contained 50 mM KCl: MES (Δ), MOPS ($\blacktriangle$), HEPES (o), EPPS ($\blacksquare$), Tricine ($\bullet$), Tris ($\blacktriangledown$), CHES ($\blacklozenge$)

Figure 28

Effect of MgCl_2 on asparaginase activity Half expanded leaves were harvested (in 50 mM Tris HCl) in the presence (○,●) or absence (△,▲) of 50 mM K^+ at the end of the dark period (solid symbols) or after 5 hours in the light (open symbols). Asparaginase activity (nmol asp/min/gm fr wt) was then assayed in the presence of varying concentrations of MgCl_2 . The experiment was repeated twice with similar results.

These results demonstrate that changes in pH, as well as the Mg^{2+} ion concentration during the light do not play a role in the light-dependent increase in asparaginase activity.

III.4 Asparaginase activity and K^+ ions

Asparaginase from developing seeds of *Pisum sativum* is a K^+ dependent enzyme (Sodek et.al. 1980) and for most of the work in this study, 50 mM KCl was added to buffers during extraction and assay of asparaginase. In half expanded leaf extracts asparaginase activity was detectable in the absence of K^+ ions, although a 3 to 8-fold (and sometimes more) increase in activity was noted with 20 to 50 mM K^+ . The K^+ dependent increase in asparaginase activity was much greater in extracts obtained during the light period (7 to 8-fold) compared with a 2 to 3-fold increase in activity observed at the end of the dark period (see figures 28, 29).

To examine whether diurnal variation of asparaginase activity was affected by K^+ ions, extracts were harvested in the presence or absence of K^+ from half expanded leaves during the dark and light periods (4 and 6 hours into the dark period, as well as 2 and 15 hours into the light period). These extracts were also desalted in the presence or absence of KCl and assayed for asparaginase activity. Diurnal variation in asparaginase activity was observed in the absence of K^+ ions (table 15). Potassium was also added back during assay to the extracts that were prepared without K^+ and an increase in activity, approaching that of the extracts in the presence of K^+ was noted. In other experiments it was observed that K^+ could be removed (by desalting, G25) and added back to asparaginase preparations during assay with approximately 75% recovery

Table 15

Diurnal variation of asparaginase activity in the absence of K^+ ions. Extracts were harvested and desalted in the presence or absence of 50 mM KCl, during the light period (2 and 15 hours) and in the dark period (20 and 22 hours in the photoperiod). Potassium was also added back to asparaginase preparations harvested in the absence of K^+ .

Time of sampling in hours in light (L), dark (D)		asparaginase activity nmol asp/min/gm. fr. wt.		
		K^+	no K^+	K^+ added back
L	2	63.2	32.1	49.9
	15	113.5	75.6	117.1
D	4	46.1	14.2	28.3
	6	54.3	13.7	18.7

of initial activity.

III.5 Asparaginase activity and some other ions

A range of NaCl concentrations (0 to 50 mM) were added to asparaginase extracts (desalted in Tris HCl without KCl); obtained at the end of the dark period and after 6 hours in the light. A slight increase in asparaginase activity was observed in extracts obtained during the light period (in the absence of K^+) at concentrations above 20 mM NaCl, while no change in activity was noted in the extracts obtained at the end of the dark period (figure 29).

Asparaginase was extracted at the end of the dark period in Tris HCl KCl, in the presence or absence of 10 mM NaF, an inhibitor of phosphoprotein phosphatases. An increase in activity was observed in the preparation extracted with NaF (asparaginase activity, extracted without NaF: 94.1 nmol asp./min/g.fr.wt., activity extracted with NaF 117.8 nmol asp./min/g.fr.wt., $n=2$). However, the addition of NaF (0 to 50 mM) to asparaginase assays extracted in Tris HCl KCl had no effect upon activity. The addition of 10 or 20 mM NaF to crude asparaginase preparations obtained during the light period in 10 mM NaF, during a 30 minute incubation period prior to assaying asparaginase, also had no effect on activity (i.e. activity decreased in the NaF treated extract at the same rate as the control).

A range of ions (at 10 mM unless otherwise noted) were added to asparaginase preparations that were extracted from half expanded leaves at the end of the dark period (centrifuged, and desalted in Tris HCl $\pm$ KCl). In the presence of KCl, no changes in asparaginase activity were noted when Ca^{2+} (1 mM), Mg^{2+} , K^+ , Na^+ (as the Cl^- salts), or PO_4^{3-} , SO_4^{2-} , Cl^- ,

Figure 29

Effect of K^+ and Na^+ on asparaginase activity Half expanded leaves were harvested at the end of the dark period (solid symbols) or after 6 hours in the light (open symbols) in the absence of KCl Asparaginase activity (nmol asp/min/gm fr. wt.) was then assayed in the presence of increasing concentrations of KCl (○,●) or NaCl (△,▲) The experiment was repeated twice with similar results

NO_2^- , NO_3^- (as the Na^+ salts), were added to asparaginase preparations (table 16). The addition of MnCl_2 reduced, while ZnCl_2 totally inhibited asparaginase activity in preparations obtained in the presence of KCl. When asparaginase was assayed with the same ions in the absence of KCl, several different responses were observed. No change in asparaginase activity was observed when PO_4^{3-} , or SO_4^{2-} (Na^+ salts) were added during assay. The additions of K^+ , Ca^{2+} (Cl^- salts), and NO_3^- (Na^+ salt), increased asparaginase activity, while Cl^- , Zn^{2+} , and Mn^{2+} reduced or inhibited activity (table 16). From these observations it appears that asparaginase is more sensitive to the effects of ions in the absence of K^+ . However, additions of ions to asparaginase obtained in the absence of K^+ did not increase asparaginase activity to the level of activity observed in the light.

III.6 Asparaginase, Ca^{2+} and calmodulin

Asparaginase was extracted at the end of the dark period and after 6 hours in the light in Tris HCl buffer $\pm$ KCl and assayed in the presence of 0 to 10 mM CaCl_2 . The addition of CaCl_2 to asparaginase ($\pm$ KCl) had little effect on activity (figure 30). When asparaginase was extracted in Tris HCl KCl containing 1 mM CaCl_2 , an increase in activity was observed compared to asparaginase extracted in Tris HCl KCl (see figure 31). Potassium was added back to asparaginase preparations that were extracted at the end of the dark period ($\pm$ 1 mM CaCl_2 , no K^+) and a 2-fold increase in activity was observed in the asparaginase preparation obtained in the presence of Ca^{2+} (figure 31). This experiment was

Table 16

The effect of ions upon asparaginase activity. Asparaginase was harvested in TrisHCl $\pm$ KCl at the end of the dark period. Assays included 10 mM ion except for CaCl_2 (1 mM), and KCl (20 mM). Values are expressed as percent initial activity (control).

Treatment	extracted in K^+		extracted without K^+	
	asnase activity ^a	% control	asnase activity ^a	% control
Control	76.5	100	5.2	100
add KCl	76.5	100	44.7	860
CaCl_2	81.0	106	9.5	183
NaCl	68.8	90	1.3	25
Na_2HPO_4	63.6	87	5.9	113
Na_2SO_4	75.4	99	4.9	94
NaNO_2	71.9	94	0	0
NaNO_3	65.0	91	10.1	192
ZnCl_2	0	0	0	0
MnCl_2	56.4	74	0	0

a. asparaginase activity (nmol asp/min/g fr. wt.)

Figure 30

Effect of CaCl_2 on asparaginase activity Half expanded leaves were harvested at the end of the dark period (solid symbols) or after 6 hours in the light (open symbols) in the presence (○,●) or absence (△,▲) of 50 mM KCl. Asparaginase activity (nmol asp/min/gm fr wt) was then assayed in the presence of increasing CaCl_2 concentrations. The experiment was repeated twice with similar results

3

MICROCOPY RESOLUTION TEST CHART
NATIONAL BUREAU OF STANDARDS
STANDARD REFERENCE MATERIAL 1010a
(ANSI and ISO TEST CHART No. 2)

Figure 31

Asparaginase activity extraction in CaCl_2 . Half expanded leaves were harvested at the end of the dark period in Tris HCl (□), or Tris HCl containing 1.0 mM CaCl_2 (○). Asparaginase activity (nmol asp/min/gm fr. wt) was then assayed in the presence of increasing concentrations of K^+ . This experiment was repeated twice with similar results.

repeated with asparaginase extracted during the light period (after 6 hours illumination) and a similar increase in activity was observed (figure 32). Kinetic analysis indicated that the increase in V_{max} for asparaginase extracted in Ca^{2+} was not accompanied by a change in the K_m for asparagine.

The removal of Ca^{2+} from asparaginase preparations reduced activity to the levels observed in Tris HCl KCl extracts and when Ca^{2+} was then added back to assays no increase asparaginase activity was observed.

Due to the effects of Ca^{2+} on asparaginase the possibility that calmodulin could mediate asparaginase activity was examined. Calmodulin is a Ca^{2+} binding protein that in the presence of Ca^{2+} becomes activated and stimulates the activity of several enzymes. A model of Ca^{2+} -calmodulin action has been proposed (Marme 1985, Roux et.al. 1986) which relates changes in Ca^{2+} in the cytosol, chloroplast, and mitochondria during the photoperiod to activated or deactivated forms of calmodulin. Small changes in the intracellular Ca^{2+} concentration can lead to large changes in calmodulin stimulated enzyme activity. To examine the possibility that calmodulin could play a role in the light-dependent increase in asparaginase activity two experiments were performed. The first involved adding trifluoperazine and chlorpromazine, inhibitors of calmodulin action, to asparaginase assays harvested (centrifuged and desalted in Tris HCl KCl and 0.5 mM $CaCl_2$) after 5 hours in the light (0.5 mM $CaCl_2$ was added to both inhibitors prior to addition in assays since these compounds bind calmodulin in a Ca^{2+} -dependent manner). No decrease in asparaginase activity was observed with either calmodulin inhibitor over a range of concentrations known to inhibit

Figure 32

Asparaginase activity: extraction in CaCl_2 at the end of the dark, or during the light periods. Half expanded leaves were harvested in Tris HCl (●) or Tris HCl containing 0.5 mM CaCl_2 (▲) at the end of the dark period (0 hour) or after 5 hours in the light (5 hour). Asparaginase activity was assayed in the presence of increasing K^+ concentrations. This experiment was repeated twice with similar results.

calmodulin-dependent processes. Instead, an increase in asparaginase activity was noted with increasing concentration of either trifluoperazine or chlorpromazine (figure 33).

The second experiment involved adding Ca^{2+} -calmodulin (commercial preparation from spinach), to asparaginase assays obtained at the end of the dark period in Tris HCl KCl ± 0.5 mM CaCl_2 . The addition of calmodulin to asparaginase preparations, in the presence or absence of CaCl_2 during extraction or assay, had no effect upon activity (control activity of asparaginase obtained $-\text{Ca}^{2+}$: 24.9 nmol asp/min/gm.fr.wt., add 500 units calmodulin in 0.5 mM CaCl_2 : 25.4 nmol asp/min/ gm.fr.wt.; control activity of asparaginase obtained $+\text{Ca}^{2+}$: 38.9 nmol asp/min/gm.fr.wt., add 500 units calmodulin in 0.5 mM CaCl_2 : 37.2 nmol asp/min/gm.fr.wt.).

These experiments indicated that an increased asparaginase activity may be achieved in the presence of Ca^{2+} under some conditions, however the response of asparaginase activity to Ca^{2+} does not appear to be mediated by calmodulin.

III. 7 Asparaginase and Phytochrome

To determine if the increase in asparaginase activity was mediated by phytochrome, soil grown plants were given 5 minute exposures to red (>650 nm), or far red (>750 nm) light, or 5 minutes of red followed with 5 minutes of far red light at the end of the dark period (see Mohr 1984), and left in the dark for a 6 hour period. A set of plants were also left in the dark without any treatment (dark control), or placed into the light over the 6 hour period (light control). Asparaginase activity was assayed from half expanded leaves obtained at the end of the dark period (0 hour),

Figure 33

Inhibitors of calmodulin action on asparaginase activity Half expanded leaves were harvested in Tris HCl containing 50 mM KCl, and 0.5 mM CaCl_2 after 5 hours in the light. Asparaginase activity (nmol asp/min/gm fr wt.) was assayed in the presence of increasing concentrations of chlorpromazine (Δ) or trifluoperzine (o). The inhibitors were prepared in 0.5 mM CaCl_2 prior to addition in the assay since they bind calmodulin in a Ca^{2+} dependant manner

and after the 6 hour treatment period. An increase in asparaginase activity was observed in the light control treatment over the 6 hour period, while a decrease in asparaginase activity was observed in the dark control treatment (table 17). Asparaginase activities extracted from plants exposed to red, far red, or red+far red light at the onset of the light period were comparable to the dark control treatment. These results indicate that the light dependent increase in asparaginase activity is not mediated by phytochrome.

III.8 Asparaginase and proteolysis

Another mechanism that could account for an increase or decrease in activity during the photoperiod is limited proteolysis of asparaginase *in vivo*. To see if asparaginase activity is modified by proteinases *in vitro*, 2 proteinases were added to asparaginase preparations, extracted at the end of the dark period and after 5 hours in the light, during assay. The addition of carboxypeptidase A (0 to 0.5 mg/ml, an exopeptidase) had no effect upon asparaginase activity obtained at the end of the dark or during the light periods, however the addition of chymotrypsinogen (an endopeptidase) over the same concentration range reduced asparaginase activity extracted in the light (figure 34). Chymotrypsinogen did not affect asparaginase activity obtained at the end of the dark period. These results indicate that limited proteolysis does not play a role in the increase in asparaginase activity during the light, however the decrease in asparaginase activity in the dark may result from proteolytic activity. If limited proteolysis of asparaginase during the dark period occurs *in vivo*, a change in the relative molecular mass (M_r) should be detectable in asparaginase extracted at the end of the dark period or

table 17

Is asparaginase mediated by phytochrome? Soil grown plants were given 5 minute exposures of red (650 nm), far red (750 nm), red+far red, or regular light at the end of the dark period and placed into the dark for 4 hour. One set of plants was placed into the light, and another kept in the dark for the same length of time. Asparaginase activity was determined at the end of the dark period (0 hr.) and after 4 hours (4 hr.)

treatment	asparaginase activity nmol asp/min/g.fr wt
0 hr.	9.7
4 hr. dark	2.3
4 hr. light	67.3
4 hr. dark following:	
5 min. red ^a	6.3
5 min. far red ^a	7.4
5 min. red+far red	8.3
5 min. reg. light	6.0

a. filters were used to supply red light > 650 nm, or far red light > 750 nm

Figure 34

Effects of proteinases on asparaginase activity. Half expanded leaves were harvested at the end of the dark period (solid symbols) or after 5 hours in the light (open symbols). Asparaginase activity (nmol asp/min/gm fr. wt.) was then assayed in the presence of increasing concentrations of carboxypeptidase A (o), or chymotrypsinogen (Δ)

during the light and this was found not to be the case (see figure 36).

When enzyme preparations were incubated at 34°C in the absence of substrate, a decrease in asparaginase activity was observed over a 30 minute period (section 1.3, figure 4). To determine if the decrease in asparaginase activity during incubation was due to proteolytic degradation of asparaginase in crude preparations, extracts were incubated with the proteinase inhibitors PMSF (10 mM, dissolved in DMSO or isopropanol) or iodoacetic acid (10 mM) for up to 60 minutes prior to assaying asparaginase activity. When PMSF in DMSO or isopropanol was incubated with asparaginase, a loss in activity below that of the control treatment (without any additions) resulted (table 18). DMSO or isopropanol alone did not decrease asparaginase activity, in fact DMSO partially stabilized asparaginase during incubation. The addition of iodoacetic acid also reduced asparaginase activity during incubation below that of the control treatment (without additions). The loss of extractable asparaginase activity in the absence of substrate during the incubation experiments does not appear to be a result of proteolytic degradation. These experiments do not preclude the possibility of total enzyme proteolysis causing a decrease in activity during the dark period *in vivo*.

III.9 Is there any soluble factor effecting asparaginase activity?

To examine the possibility that a soluble inhibitor or activator in crude extracts might modify asparaginase activity, plants were grown on different photoperiod schedules so that half expanded leaves could be obtained at the end of a dark period or after 6 hours of light at the same time. These samples were ground separately or together (coharvested) in

Table 18

Effect of PMSF and iodoacetic acid on asparaginase. Asparaginase was harvested in Tris HCl KCl CaCl_2 buffer, desalted, and incubated at 34°C for 30 or 60 minutes in 10 mM PMSF (dissolved in either DMSO or isopropanol) or iodoacetic acid. An asparaginase preparation without any additions was incubated alongside (control). Values are expressed as percent initial activity.

Treatment	% initial activity	
	30 minutes	60 minutes
Control	42	24
Iodoacetic acid	0	0
DMSO	53	45
Isopropanol	38	30
PMSF in DMSO	30	0
PMSF in isopropanol	0	0

50 mM Tris HCl, pH 8.0, 50 mM KCl, 1 mM CaCl_2 , 1 mM DTT, or the same buffer with the addition of 10 mM asparagine. Extracts were incubated for 15 minutes at 22°C, centrifuged, desalted and assayed for asparaginase.

Initial experiments indicated that there was substantial variation between asparaginase activity prepared from small batches of leaves, in both light or dark leaf extracts. To reduce this problem each pea leaf was split into 2 halves; 1 half for control asparaginase activity, and the other for coharvested activity and in this way variation was kept to a minimum between treatments. Values for each experiment were compared with that of the light extract, which was set at 100%. If a soluble factor was present in the dark or light extract, the activity obtained from the coharvested mixture would be expected to deviate from an average value of the two extracts and should approach the levels of activity of either the light or dark extract. If no change in asparaginase activity occurred in the coharvested mixture this would indicate that no soluble factors (activators or inhibitors) were present in the extracts. The experiment was repeated 4 times and no changes in asparaginase activity were observed in the coharvested mixture (table 19). The presence or absence of 10 mM asparagine did not effect the outcome of the experiment nor did the removal of DTT, or the addition of 10 mM NaF during extract preparation and incubation.

These results indicate that a soluble factor does not account for the change in asparaginase activity during the photoperiod.

III.10 Asparaginase, gel filtration and ion exchange chromatography

The relative molecular mass (M_r) of asparaginase extracted from

Table 19

Effect of coharvesting on asparaginase activity. Half expanded leaves were obtained at the end of the dark period (0 hr.) or after 6 hours of light (6 hr.) and ground separately or together on a fresh weight basis (0+6 hr.) in (A) Tris HCl KCl with 1 mM CaCl_2 , 1 mM DTT, or in (B) Tris HCl KCl CaCl_2 DTT and 10 mM asparagine, centrifuged, desalted and assayed. Values are expressed as a percentage of light activity.

Treatment		% light activity	±SD
A			
0 hr.	D	21	8.3
6 hr.	L	100	-
0+6 hr.	D+L	65	13.0
(expected avg of mix)		60.5	-
B +ASN			
0 hr.	D	19	5.3
6 hr.	L	100	-
0+6 hr.	D+L	61	16.0
(expected avg of mix)		59.5	-

developing *Lupinus polyphyllus* or *Pisum sativum* seeds is 68000 to 75000 (Lea and Mifflin 1978, Chang and Farnden 1981, Sodek et. al. 1980). Since SDS gel electrophoresis of the Lupin enzyme indicated that asparaginase was a dimer (subunits Mr 38000, Lea and Mifflin 1978), the possibility that a change in the Mr of half expanded pea leaf asparaginase, due to aggregation or dissaggregation of subunits that could take place during the photoperiod, was examined using gel filtration (S300) chromatography. Another mechanism that might account for a modification of asparaginase activity is that a change in the charge of the enzyme (eg. due to phosphorylation or dephosphorylation of asparaginase) could take place during the photoperiod, and this was examined by using DEAE cellulose chromatography.

Asparaginase preparations were obtained at the end of the dark period or after 7 hours in the light and passed through columns of S300 or DEAE cellulose in order to determine whether post-translational modifications could account for the difference in asparaginase activity. Enzyme precipitates from 25 to 65% $(\text{NH}_4)_2\text{SO}_4$ fractions, or crude enzyme preparations after an initial 20000 *g* centrifugation (no salting out), gave similar results on S300 columns. Only one peak of asparaginase activity was recovered (approximately 14 to 15-fold purified) after S300 chromatography. No difference was observed in the relative molecular mass of asparaginase extracted at the end of the dark or during the light period (figure 35). The relative molecular mass of asparaginase from half expanded pea leaves was estimated at 68000 (figure 36).

Crude enzyme preparations were desalted (G25), placed onto DEAE cellulose columns and eluted with KCl (0.05 to 0.4 M) gradients. Only one

Figure 35

Gel permeation (Sephacryl S300) chromatography of asparaginase. Half expanded leaves were harvested at the end of the dark period (solid symbols) or after 7 hours in the light (open symbols). Precipitates obtained from a 25 to 65 % $(\text{NH}_4)_2\text{SO}_4$ treatment were applied onto a S300 column (2.6 cm x 80 cm) and eluted in extraction buffer with a flow rate of 20 ml/hour. Asparaginase activity ($\Delta, \blacktriangle$) was assayed by measuring NH_3 production and is expressed as A_{420} (the wavelength maximum of Nessler's reagent). Protein ($\square, \blacksquare$) was monitored at A_{280} .

Figure 36

Relative molecular mass of asparaginase. Asparaginase was passed through a Sephacryl S300 column (2.6 x 80 cm) previously calibrated with Blue dextran (Mr. 200,000, to determine the void volume of the column: V_0), alcohol-dehydrogenase (ADH, Mr. 150,000), bovin serum albumin (BSA, Mr. 66,000), carbonic anhydrase (CA, Mr. 29,000), and cytochrome C (cyt C, Mr. 12,400). The elution volume (V_e) of each protein was plotted relative to void volume and is expressed as a ratio: V_e/V_0 . The Mr. of pea leaf asparaginase obtained at the end of the dark period, or during the light period was estimated at 68,000.

Figure 37

DEAE Cellulose chromatography of asparaginase. Half expanded leaves harvested at the end of the dark period (solid symbols) or after 7 hours in the light (open symbols) were desalted (through Sephadex G 25) and applied to columns (1.0 x 26 cm, flow rate 20 ml/hour) of DEAE cellulose. After the extracts were loaded and washed with extraction buffer, gradients of 0.05 to 0.2 M KCl (200 ml) were applied (solid line). Asparaginase activity ($\Delta, \blacktriangle$) was assayed by measuring NH_3 production (A_{420}), and protein (broken line) was monitored at A_{280} .

peak of asparaginase activity was obtained at both the end of the dark period or during the light, and these peaks of activity co-eluted at approximately 0.21 M KCl (figure 37). These results indicate that post translational changes do not account for the increase in asparaginase activity upon illumination.

During gel filtration experiments it was observed that the total activity in asparaginase preparations, extracted during the light, increased after S300 chromatography, when compared to the total activity of crude extract (asparaginase activity in crude extract: 100%, and activity after S300 $141\% \pm 18$, $n=3$). Since no increase in total asparaginase activity was observed when preparations obtained at the end of the dark period were treated with S300 (crude activity 100%, and after S300 $92\% \pm 15$, $n=3$), it appeared that an inhibitor (removed by S300) might be present in the light. However it has already been shown (section III.9) that there is no evidence for the presence of an inhibitor or activator. The reason for activation of asparaginase activity obtained in the light after S300 is not clear.

III.11 Asparaginase: membrane bound vs free enzyme

If asparaginase was membrane bound in the dark and soluble in the light, then it would remain in the particulate fraction during extraction at the end of the dark period, and since centrifuged fractions were routinely used for asparaginase assays, preparations harvested at the end of the dark period might therefore yield lower asparaginase activity. To determine if changes in extractable asparaginase activity during the photoperiod could be due to association and disassociation between the enzyme and membrane fractions, experiments were performed upon

preparations obtained at the end of the dark period.

Half expanded leaves were ground in Tris HCl buffer, centrifuged at 200 *g* for 2 minutes to remove cellular debris and the supernatant was recentrifuged at 20000 *g* for 10 minutes. The pellets from the 20000 *g* centrifugation were resuspended in one of the following 4 buffers, which might be expected to release bound enzyme: 1) 50 mM Tris HCl, pH 8.0, 50 mM KCl, 2) Tris HCl KCl containing 0.1 % triton, 3) Tris HCl KCl containing 1 mM CaCl_2 , or 4) 50 mM potassium phosphate, pH 8.0, containing 20% glycerol; an aliquot of each treatment was immediately removed, centrifuged at 20000 *g* and assayed for initial asparaginase activity. As a control for these buffers, the supernatant from the initial extract was split into four groups, desalted in the same buffers as above, and an aliquot removed from each treatment and assayed for initial activity.

Supernatants and pellet extractions were incubated on ice for 1 hour and afterward assayed for asparaginase activity (over this period of time no appreciable loss of asparaginase was detected, if extracts were maintained on ice, section I.2). The particulate treatments were centrifuged and the supernatant assayed for asparaginase. Only slight changes in asparaginase activity were observed in the soluble, or the particulate fraction treatments during the 1 hour incubation period (table 20). Therefore association and disassociation of asparaginase and a membrane or particulate fraction does not appear to explain the changes in extractable activity.

III.12 Asparaginase and protein synthesis

The diurnal change in asparaginase activity was observed when

Table 20

Is asparaginase membrane bound? Half expanded leaves were removed at the end of the dark period, ground in Tris HCl buffer, centrifuged at 200 *g* and the supernatant centrifuged again at 20000 *g*. The particulate fractions of the second centrifugation were extracted in four different buffers; the 20000 *g* supernatant (soluble fraction) was desalted in the same series of buffers. Both fractions were assayed before and after a 1 hour incubation period on ice (see text for details).

treatment	nmol asp/min/mg protein		
	initial activity	1 hour incubation	Δ activity
Tris HCl	0.53	0.5	-0.03
soluble fraction			
Tris HCl KCl	0.75	0.80	+0.05
Tris HCl KCl CaCl ₂	0.89	0.85	-0.04
Tris HCl KCl 0.1% triton	0.92	0.87	-0.05
Potassium phosphate 20% glycerol	1.11	1.13	+0.02
particulate fraction			
Tris HCl KCl	0.29	0.11	-0.18
Tris HCl KCl CaCl ₂	0.23	0.30	+0.07
Tris HCl KCl 0.1% triton	0.16	0.24	+0.08
Potassium phosphate 20% glycerol	0.31	0.28	-0.03

measured using $(\text{NH}_4)_2\text{SO}_4$ -precipitated as well as desalted enzyme preparations. If the activity change is due to alteration of the properties of an existing enzyme, then the changed state persists through desalting and protein precipitation (or a factor remains tightly bound). An alternative explanation of the changed activity could be that asparaginase is synthesized *de novo* during the light period. To examine whether protein synthesis might play a part in the light dependent increase in asparaginase activity, 2 inhibitors of transcription, and 4 inhibitors of organelle or cytosolic translation were fed to half expanded leaves at the end of the dark period for 3.5 hours in the light.

In the experiments to determine the effects of protein synthesis inhibitors on asparaginase activity, feeding solutions contained 8 mM asparagine, 0.17 μCi (0.06 MBq) ^{14}C -leu and inhibitor (all at pH 6.3). Asparaginase activity was determined at the beginning and end of feeding experiments, and protein synthesis (incorporation of ^{14}C -leu into ethanol insoluble precipitates) during the treatment was measured.

During the 3.5 hour feeding period protein synthesis and an increase in asparaginase activity took place in the control treatments containing asparagine and ^{14}C -leu (figure 38, table 21). After feeding the 6 protein synthesis inhibitors, 3 trends in asparaginase activity during the feeding experiment were observed: cycloheximide, cordycepin and actinomycin D inhibited the increase of asparaginase activity (compared with the control treatment) for all concentrations tested; emetine and puromycin increased asparaginase activity at low concentrations and decreased activity at higher concentrations; all concentrations of chloramphenicol increased asparaginase activity.

Figure 38

Effects of inhibitors of protein synthesis on asparaginase activity. Half expanded leaves were harvested at the end of the dark period (initial asparaginase activity: solid symbol) and fed for 35 hours in the light (open symbols). Feeding solutions contained 8 mM asparagine and increasing concentrations of chloroamphenicol ($\square$), puromycin (Δ), emetine ($\circ$), actinomycin D ($\circ$), cordycepin ($\diamond$), and cycloheximide (∇). Values are expressed as percent control asparaginase activity. All of the treatments were repeated twice with similar results.

chloramphenicol □, puromycin Δ, actinomycin D ○

cordycepin ◇, cycloheximide ▽, emetine ⊙

Table 21

Effect of protein synthesis inhibitors on asparaginase activity
 Half-expanded leaves were fed a range of inhibitors of protein synthesis for 3.5 hours in the light, starting at the end of the dark period. Feeding solutions contained 8 mM asparagine, ^{14}C -leu, and inhibitor (all at pH 6.3). At the end of the dark period (0hr.) and after 3.5 hours in the light (3.5 hr) asparaginase activity and protein synthesis (incorporation of ^{14}C -leu into ethanol insoluble precipitates) were determined. Values are expressed as percentage of asparaginase or protein synthesis values at 3.5 hours.

Treatment		asparaginase activity (% 3.5 hour activity)	protein synthesis (% 3.5 hour ^{14}C -leu incorporation)
	$\mu\text{g/ml}$		
0 hr		38	0
3.5 hr		100	100
cycloheximide	1	55	46
actinomycin D	10	64	65
	50	14	49
cordycepin	10	83	72
	50	46	53
emetine	50	110	83
	100	74	73
puromycin	50	130	91
	200	42	88
chloramphenicol	20	106	97
	100	138	95

The addition of cycloheximide at 1 $\mu\text{g/ml}$ inhibited the light dependent increase in asparaginase activity, while at the same time reduced protein synthesis by 45%. With increasing cycloheximide concentrations in the feeding solutions, asparaginase activity was lower after the 3.5 hour feeding period in the light than the initial activity at the end of the dark period. Low concentrations (10 $\mu\text{g/ml}$) of cordycepin and actinomycin D also prevented the light dependent increase in asparaginase activity, and at higher concentrations asparaginase activity decreased below that of the level observed at the end of the dark period.

Emetine, puromycin and chloramphenicol all increased asparaginase activity below 50 $\mu\text{g/ml}$ of inhibitor and at these inhibitor concentrations protein synthesis decreased about 5 to 20%. At higher inhibitor concentrations protein synthesis was inhibited from 10 to 30% and emetine and puromycin abolished the increase in asparaginase observed in the control treatments, although asparaginase activity approximated the level of activity observed at the end of the dark period. Chloramphenicol stimulated asparaginase activity during the feeding experiment over all concentrations tested, while protein synthesis decreased by only 5% at 100 $\mu\text{g/ml}$, the highest concentration tested.

These results indicate that protein synthesis may play an important role in the light dependent increase in asparaginase activity.

It has been reported that feeding cycloheximide (1.0 $\mu\text{g/ml}$) or puromycin (50 $\mu\text{g/ml}$) for 4 hours to cultured rose cells led to increased NH_3 levels, above those that have been demonstrated to be toxic to plants (Bradford and Fletcher 1982). These authors suggested caution when interpreting results from experiments that involve feeding protein

synthesis inhibitors due to the possibility of NH_3 toxicity leading to secondary effects. Therefore the levels of NH_3 were examined in leaf homogenates after feeding cycloheximide (5 μg), puromycin (50 and 200 μg), or actinomycin D (10 and 50 μg) for 3.5 hours in the light. The levels of NH_3 in leaves fed protein synthesis inhibitors were approximately the same as levels of NH_3 determined in leaves obtained either at the end of the dark period, or in the control treatment after 3.5 hours of light (values ranged within 0.97 to 1.6 $\mu\text{g NH}_3/\text{g fr. wt.}$ for all treatments). No increase in NH_3 levels were observed in leaf homogenates after the inhibitor feedings.

Since photosynthesis is required for the increase in asparaginase activity (section II.3), it was necessary to determine whether the inhibition of protein synthesis had an effect upon photosynthetic activity. Several inhibitors of protein synthesis were fed to half expanded leaves for 3.5 hours in the light and photosynthetic activity (measured by DCPIP reduction) was determined in crude chloroplast membrane preparations. No change in photosynthetic activity was observed after emetine (50 and 100 μg), puromycin (50 and 200 μg) or cordycepin (10 and 50 μg) were fed to half expanded leaves, compared with photosynthetic activity assayed at the end of the dark period, or in the control treatment (for all treatments the change in DCPIP reduction (ΔA_{610}) was within 6.2 to 7.6 $\Delta A_{610}/\text{min/mg chlorophyll}$).

In section I.10 results were presented that indicated that asparaginase activity did not increase during feeding experiments in half expanded leaves obtained from hydroponic plants. Since the above

results indicate that protein synthesis may play a role in the increase of asparaginase activity, ^{14}C -leu was fed to half expanded leaves of hydroponic and soil plants (obtained at the end of the dark period for 3 hours in the ~~light~~) in order to establish whether the decrease in asparaginase activity in hydroponic leaves may be a result of reduced protein synthesis. After 3 hours of feeding, ^{14}C -leu incorporation into protein was lower in detached leaves from hydroponic plants but substantial protein synthesis had taken place eventhough no increase in asparaginase activity was observed (^{14}C -leu incorporation in soil grown leaves 80.7×10^3 cpm/3 hr./g.fr.wt., asparaginase activity increased 2-fold; ^{14}C -leu in hydroponic leaves 60.0×10^3 cpm/3 hr./g.fr.wt., asparaginase activity decreased by a third). This result indicates that the decrease in asparaginase activity in hydroponic leaves was not due to reduced protein synthesis.

Discussion

NH₃ determination by microdiffusion

During this study, routine measurements were required for the estimation of NH₃ levels in leaf samples and in deamidation reactions. A number of methods have been described for NH₃ determination in biological fluids or enzyme preparations including enzymatic determination of NH₃ with glutamate dehydrogenase (Cheuk and Finne 1984, Gella and Pascual 1982, Nazar and Schoolwerth 1979), or direct colorimetric determination of NH₃ by nesslerization (Tate and Meister 1975, Imada et al. 1973, Wade et al. 1971, Koch and McKeekin 1924) or phenol hypochlorite (Patton and Crouch 1977, McCullough 1967, Muftic 1964, Russell 1944). These methods were found to be unsuitable for routine use in the present study since compounds such as DTT, glyoxylate, isonicotinyl hydrazide, a range of cations and anions, and the amides glutamine and asparagine interfered with these NH₃ assays. Direct colorimetric approaches using nesslerization or the indophenol reaction (Berthelot reaction) have been previously found unsatisfactory due to interference by substrates (e.g. with glutamine, Gella and Pascual 1984, Tate and Meister 1975, Curthoys and Lowry 1973; nicotinamide, Chaykin 1969; or ions, Muftic 1964), insensitivity (Nazar and Schoolwerth 1979, Tetlow and Wilson 1964) and poor reproducibility (Bolleter et al. 1961). Methods such as Conway diffusion (Conway and O'Malley 1942), or microdiffusion (a procedure using smaller volumes than Conway diffusion, described by Burris 1972) have been criticized

due to the length of time involved (3 hours per assay, Chaykin 1969, Nazar and Schoolwerth 1979), hydrolysis of alkali-labile compounds during diffusion (McCullough 1967), or 'lack of accuracy' (Mizobuchi et al. 1984).

Alternate methods of NH_3 estimation include the NH_3 electrode (Dewolfs et al. 1975), or HPLC (Mizobuchi et al. 1984). These methods were not suitable for use in this study for several reasons. A major drawback with the NH_3 electrode was that it required large sample volumes for NH_3 analysis (50 ml, Dewolfs et al. 1975). Analysis of NH_3 by HPLC was not suitable for large sample numbers due to the time needed for analysis per sample, for example when assaying column profiles. The procedure of microdiffusion (Burris 1972) was therefore reexamined as a possible alternative in conjunction with nesslerization or phenol hypochlorite for the detection of NH_3 .

Nesslerization was found to be a reliable method for the determination of NH_3 and the reagent more convenient to prepare and use than phenol hypochlorite. The reagent as prepared by the method of Vanselow (1940) was stable, easy to prepare and use, and sensitive in the nmol (NH_3) range. Earlier methods for the preparation of Nessler's reagent were rather involved (e.g. Koch and McMeekin 1924, Folin and Wu 1965), and caution was required during use because of slow colour development and precipitate formation, an observation confirmed during the present study. However, Vanselow (1940) reported that increasing the concentration of KOH during preparation of the reagent increased the rate of colour formation in the presence of NH_3 and reduced precipitate

formation in storage and use.

Time-course studies during microdiffusion indicated that the diffusion of NH_3 from the K_2CO_3 solution (1 ml volume in stoppered scintillation vials) was complete in less than 60 minutes (figure 1), shorter than the 3 hour time interval reported previously (Burris 1972). Recovery of NH_3 from the microdiffusion vials was linear to at least 200 nmol NH_3 (linear up to 800 nmol NH_3 with $(\text{NH}_4)_2\text{SO}_4$ as a standard, Shelp et al. 1985), and values for standards were similar to those obtained from direct nesslerization (figure 1). Using microdiffusion, nonenzymatic NH_3 production from amides in K_2CO_3 solution was much less than in Nessler's reagent. Rates of deamidation in K_2CO_3 for asparagine were 0.2%/minute, and 0.6%/minute for glutamine. With direct nesslerization, asparagine was deamidated at a rate of 0.9%/minute and glutamine at 15.5%/minute. The rate of nonenzymatic glutamine deamidation in Nessler's reagent approximates that reported earlier (Tate and Meister 1975). The procedure of microdiffusion followed by nesslerization overcame many of the problems discussed above; absorbance values were stable, error introduced by time differences between reading samples was minimal, interference from other solutes during microdiffusion or colour development was negligible, and handling large sample numbers (50 to 60 samples) was not difficult. However, it was found that this technique was not suitable for kinetic analysis since background levels of NH_3 fluctuated. For routine assays, samples were run in triplicate to overcome possible interference of background NH_3 levels.

Properties of asparaginase from developing pea leaves

Asparaginase extracted from developing fifth leaves was less stable during storage than the enzyme from developing cotyledon tissue (figure 3) and shows other differences from the seed enzyme. It has been reported that in lupin, asparaginase obtained from leaves was also less stable than the cotyledon asparaginase (Chang and Farnden 1981). The pea leaf enzyme required K^+ as do many other plant asparaginases, although young leaf asparaginase activity was detectable in the absence of K^+ (tables 15, 16) in contrast to reports of absolute K^+ dependancy for the enzyme from developing pea cotyledon (Sodek et al. 1980). Sodek et al. (1980) also reported that K^+ (50 mM) helped stabilize asparaginase obtained from pea cotyledons, since no loss of activity was observed over a 60 minute period at 20 or 30°C in the presence of K^+ . They suggested that K^+ may help maintain the functional integrity of the enzyme because in the absence of K^+ asparaginase was unstable, possibly dissociating into two inactive subunits, however, no evidence for this mechanism was provided. When cotyledon asparaginase was incubated in the presence of asparagine (10 mM) a decrease in activity was observed (Sodek et al. 1980). In the present study, asparaginase activity obtained from pea leaves decreased rapidly *in vitro* even in the presence of K^+ , and activity was only maintained in the presence of asparagine (10 mM), aspartate (6 mM), glycerol (20%), and was partially protected by PO_4^{3-} (10 mM) and DTT (>10 mM) (figures 4, 5, table 12, 13).

Purified asparaginase II from *Saccharomyces cerevisiae* was

stable *in vitro*, although the enzyme was sensitive to SO_4^{2-} , and a decrease in activity over a 60 minute period was observed in the presence of this ion. The addition of asparagine (10 mM) to asparaginase II preparations in the presence of SO_4^{2-} (10 mM) partially protected activity (Kim and Roon 1983). In the present study, SO_4^{2-} had no effect on asparaginase activity (table 16).

The differential stability of asparaginase at 34°C with varying asparagine concentrations (figure 4) is worthy of comment since it indicates that at low asparagine concentrations asparaginase is not only less active, but that the enzyme is also less stable. Therefore, the apparent K_m s for asparagine of 7.1 and 15.9 mM (section III.1, and figure 24) need to be treated with caution - since they were obtained over a range of concentrations, they may not be true representations of the affinity of asparaginase for asparagine. However, the estimated K_m s for the leaf enzyme approximate the K_m s for asparagine from other plant asparaginases; i.e. 3.0 mM for pea cotyledon (Sodek et al. 1980, Ireland and Joy 1981), 6.6 to 7.0 mM for lupin cotyledon (Chang and Farnden 1981), 8.0 mM for pea leaf (Ireland and Joy 1981), and 12.2 mM for lupin cotyledon (Lea et al. 1978) asparaginases.

Aspartate also stabilized asparaginase activity during incubation (figure 5), but asparaginase activity was inhibited about 25% with 10 mM aspartate in the assay (and 20 mM asparagine, data not shown). This is similar to the aspartate inhibition of pea cotyledon asparaginase (Sodek et al. 1980), however, lupin cotyledon enzyme was not inhibited by aspartate (Lea et al. 1978). Difficulties were encountered in trying to

obtain an accurate K_m value for aspartate (estimated at 12 mM). High values of background aspartate levels in the assay introduced error upon analysis of aspartate by HPLC, and as mentioned above, the NH_3 assay was not suitable for kinetic analysis. Ammonia (up to 10 mM) did not inhibit the reaction, and this is similar to the response observed for cotyledon asparaginases: no inhibition of asparaginase activity (K^+ -dependant cotyledon enzyme) was observed with 25 mM NH_3 (*Lupinus arboreus* or *L. angustifolius*, Chang and Farnden 1981), and only 6% inhibition of activity was observed at 10 mM NH_3 (K^+ -independant cotyledon asparaginase from *L. polyphyllus*, Lea et al. 1978). Ammonia did not stabilize pea leaf asparaginase activity in the present study (figure 5).

The response of asparaginase activity to a range of ions (figures 28, 29, 30, table 16) suggested that in the absence of K^+ , asparaginase was more sensitive to CaCl_2 , NaNO_3 , NaNO_2 , MgCl_2 , and MnCl_2 than it is in the presence of K^+ . What this might mean *in vivo* is not clear unless changes in the concentration of K^+ and other ions do take place throughout the photoperiod (as has been suggested by Demmig and Gimmler 1983, Moore and Akerman 1984, Kreimer et al. 1985). However, estimates of K^+ concentrations within the cytosol and chloroplast vary from 70 to 180 mM (Flowers and Lauchli 1983, Wyn Jones and Pollard 1983, Robinson and Downton 1984), and are well above the 20 mM K^+ concentration required to activate asparaginase (e.g. figure 31). This therefore suggests that alterations of asparaginase activity by ions do not play a role *in vivo*. It is also of interest to note that diurnal variation of asparaginase activity in half expanded leaves was observed

when extracts were obtained in the presence or absence of K^+ (table 15).

The addition of Ca^{2+} to lupin cotyledon (35 mM Ca^{2+} , Lea et al. 1978), or pea cotyledon (33 mM Ca^{2+} , Sodek et al. 1980) asparaginase had no effect on activity, nor did Ca^{2+} (0.5 to 10 mM) affect pea leaf asparaginase (figure 30). However, when pea leaf asparaginase was extracted and maintained in the presence of Ca^{2+} (0.5 mM), 2-fold higher activity was obtained (figures 31, 32). Extraction of developing pea cotyledon at three different ages in the presence of Ca^{2+} had no effect on asparaginase activity when compared with the minus Ca^{2+} extracts (data not shown). The higher levels of asparaginase activity in pea leaf extracts due to Ca^{2+} does not appear to be controlled by Ca^{2+} -calmodulin. Inhibitors of calmodulin action, trifluoperazine and chlorpromazine, at concentrations known to inhibit calmodulin regulated processes (Veigl et al. 1984) did not decrease asparaginase activity in the presence of Ca^{2+} , rather, they gave an increase in activity (figure 33). The addition of Ca^{2+} -calmodulin (spinach calmodulin) to crude pea leaf preparations also had no effect on asparaginase activity (section III.6).

The activities of only a few enzymes are currently known to be directly affected by Ca^{2+} (however, Ca^{2+} in conjunction with calmodulin may affect many cellular processes including protein kinases, ATPases, and cellular NAD^+ - and $NADP^+$ - levels, e.g. Marme 1985). Direct effects by calcium on enzyme activity have been observed with mitochondrial GDH (Joy 1973, Furuhashi and Takahashi 1982), external-mitochondrial NADH dehydrogenase (Moore and Akerman 1984), cell wall glucan synthase (Kauss et al. 1983), and chloroplast FBPase and SBPase (Hertig and Wolosiuk 1980, Wolosiuk et al. 1982). For all of these enzymes, except FBPase and SBPase, activity was stimulated in the presence of

low (≤ 0.5 mM) Ca^{2+} concentrations.

The role of Ca^{2+} in modifying chloroplast FBPase and SBPase activities is more complicated. At micromolar concentrations, Ca^{2+} enhanced activation of FBPase (in the presence of fructose 1,6-bisphosphate, DTT, and reduced thioredoxin, Hertig and Wolosiuk 1980), however Ca^{2+} also inhibited the catalytic activity of FBPase (Charles and Halliwell 1980, Hertig and Wolosiuk 1980). Calcium also enhanced (Wolosiuk et al. 1982) and inhibited (Charles and Halliwell 1980, Wolosiuk et al. 1982) SBPase activity in a similar manner. The enhancement of activation of FBPase and SBPase in the presence of Ca^{2+} , substrate, and DTT was also observed in the absence of DTT, however the final activity of both enzymes was lower than if DTT was present during activation (Hertig and Wolosiuk 1980, Wolosiuk et al 1982). The addition of Ca^{2+} to pea leaf asparaginase preparations, in the presence or absence of DTT or asparagine had no effect on activity (section III.2, tables 12, 13). The significance of increased asparaginase activity when extracted and maintained in the presence of Ca^{2+} is not clear.

Asparaginase activity in the presence or absence of K^+ was inhibited by 10 mM Zn^{2+} , the only concentration examined (table 16). Inhibition of plant asparaginase by Zn^{2+} has not been previously reported, however Lea et al. (1978) observed that heavy metals (e.g. Hg^{2+} , Fe^{3+} , Co^{2+} , Ni^{2+} all at 8.3 mM) inhibited cotyledon (K^+ -independent) asparaginase. Nitrate reductase and GDH are also inhibited by heavy metals. For example, Fe^{3+} , Cu^{2+} , and Zn^{2+} were observed to reversibly inhibit NR activity (Smarrelli and Campbell 1983), and Zn^{2+} at low concentrations (0.3 mM) stimulated GDH activity, while at higher concentrations (above 1.0 mM) inhibited activity (Joy 1973, Stewart et al. 1980).

Heavy metals are reported to inhibit or have no effect on microbial asparaginase activity. At 1.0 mM, Zn^{2+} inhibited asparaginase activity by 40% in *Actinetobacter calcoaceticus* (Joner et al. 1973), but at 5 mM, ZnCl_2 had no effect on the enzyme from *S. cerevisiae* (Kim and Roon 1983). Asparaginase inactivation by a Zn^{2+} -dependant (ZnSO_4) protease was proposed as a novel method for 'down-regulation' of the enzyme in *S. cerevisiae* (Pauling and Jones 1980, in Kim and Roon 1983). However, proteolysis does not appear to account for the decrease in pea leaf asparaginase activity observed *in vitro* since the addition of protease inhibitors, PMSF and iodoacetate (section III.8), or non-hydrolyzed casein (1%; table 13) to extracted preparations had no effect on the subsequent decrease of asparaginase activity.

The suggestion that yeast asparaginase might be down-regulated by a Zn^{2+} dependant protease was later questioned by Kim and Roon (1983) for several reasons including 1) the kinetics of inactivation of purified and crude asparaginase preparations were not due to Zn^{2+} , but rather SO_4^{2-} additions to the assays, and 2) inactivation was independant of EDTA (5 mM, a divalent cation chelator) addition. The decrease in pea leaf asparaginase during a 30 minute incubation period (figure 4) was not affected by the addition of EDTA (1 and 5 mM, table 13), nor was it affected by extracting and incubating asparaginase in 1 mM EDTA (data not shown).

The relative molecular mass (M_r) estimated for pea leaf asparaginase (M_r 68,000, using S300, gel permeation chromatography, figure 36) approximates those reported for pea cotyledon asparaginase (M_r 68,300, Sodek et al. 1980), and lupin cotyledon asparaginase (M_r 72,000, Lea et al.

1978, Mr 75,000, Chang and Farnden 1981). Only one peak of pea leaf asparaginase activity was observed after DEAE cellulose (figure 38) or hydroxylapatite (data not shown) chromatography suggesting that no other isoform of asparaginase exists. The pH optimum for plant, and several microbial, asparaginases is broad (Wriston 1985) and this appears to hold true for pea leaf asparaginase as well (figure 27).

Diurnal variation of asparaginase activity: leaf and plant age

Asparaginase activity must be regulated in some manner since asparaginase activity was detected in developing leaves, but was absent in mature leaves. Furthermore, as each young leaf developed, it showed a diurnal variation in asparaginase activity during a three day period early in its growth (figure 10). During this period, a rapid increase in the fresh and dry weight, total nitrogen, and chlorophyll content of each leaf also takes place (Urquhart and Joy 1982). When the leaf is approximately half expanded (stages 4 to 5), the most pronounced rate of increase in nitrogen content of the leaf is noted and it is at this stage of leaf development that maximum diurnal variation of asparaginase activity is observed (figure 10). Stage 3 leaves exhibited about a 2 to 3 fold increase in asparaginase activity during the photoperiod (and a corresponding decrease in the dark), while increases (and decreases) of activity up to 5 fold were noted in half expanded leaves (stage 5, figure 10, table 1). The increase of activity during the light period, and decrease of activity in the dark, was observed when expressed on a fresh weight basis, a mg protein basis, and on a per leaf basis, indicating that the changes in asparaginase activity were not a result of changes in fresh weight or leaf growth during the photoperiod.

Diurnal variation in asparaginase activity increased in successive stage 3 and stage 5 leaves as plants aged (table 1), indicating that asparaginase activity is not the same (on a fresh weight or protein basis) for each young or half expanded leaf during plant development. As a result, maximum diurnal variation of asparaginase activity was observed in half expanded, fifth or sixth leaves (counting from the bottom) on the pea plant.

It is of interest to note that an increase in asparaginase activity with plant age, both at the end of the dark, and during the light periods (table 1), coincided with a three-fold decrease in total organic nitrogen levels in the xylem exudate (figure 13, table 4). The decrease of nitrogen in xylem exudates during plant development is accounted for by a decrease in several amino compounds, but mostly by a decrease in the levels of asparagine and homoserine. A similar decrease in levels of asparagine were also noted in young and half expanded leaves (figure 14, tables 2 and 3). It is tempting to speculate that gross levels of leaf asparaginase may be regulated by nitrogen catabolite repression (NCR) in an analogous manner to that observed for extracellular asparaginases in microorganisms (e.g. Dunlop and Roon 1975, 1980, Paul and Cooksey 1981a). With this form of regulation, nitrogen depletion derepresses (induces) the synthesis of asparaginase and several other enzymes, however the mechanism(s) regulating NCR are not known (Dunlop et al. 1982). Increased levels of asparaginase, as incoming and internal levels of asparagine fall while there is still a demand for nitrogen, may be a similar response.

A different situation has been observed for leaf NR and NiR activities since during plant development increased activities (e.g. Kakefuda et al.

1983, Gupta and Beevers 1985) and increased enzyme levels (Somers et al. 1983, Gupta and Beevers 1985) have been demonstrated in the presence of NO_3^- .

The supply of nitrogen from asparagine during leaf development appears to be a complex process. In young and half expanded pea leaves, asparaginase activity is high, however, asparaginase activity decreases with leaf age and reaches low levels of activity about one day before the leaf is fully expanded (Ireland and Joy 1981). Since asparagine-nitrogen would still be required for amino acid and protein synthesis, the route of asparagine catabolism changes from predominantly hydrolysis (via asparaginase), to transamination (Ireland and Joy 1981, Ta and Joy 1985). A decrease in asparaginase activity, and increased asparagine aminotransferase activity with leaf age, has also been reported in developing lupin leaves (Atkins et al. 1983).

Effects of light and dark on asparaginase activity

Several experiments demonstrated that light was required for the increase of asparaginase activity (section II). Diurnal variation of asparaginase activity was not an endogenous rhythm since the variation in activity was not continued when the light and dark periods were extended. This is unlike the situation for NR (and other enzymes involved in nitrogen metabolism e.g. GDH and GS, see introduction) which displayed a circadian rhythm in activity during prolonged light or dark periods (e.g. Duke et al. 1978, Lillo 1984b).

Asparaginase activity from half expanded pea leaves increased with increasing energy fluence rates over a 5 hour period (figure 16, 17). An increase in extracellular asparaginase activity from *Chlamydomonas*

also coincided with increasing energy fluence rate, however an illumination period of about 36 hours was required to obtain a 4-fold increase in activity (Paul and Cooksey 1981a). Induction of *Chlamydomonas* asparaginase by light was only observed in the absence of nitrogen since derepression of synthesis is regulated by NCR (Paul and Cooksey 1979, 1981b), and the addition of 5 mM NH_4Cl abolished the light dependant increase in activity (Paul and Cooksey 1981a). Nitrate reductase activity has also been shown to be dependant upon energy fluence rate (Srivastava 1980, Duke and Duke 1984, Lillo 1984c), and required approximately 4 hours to increase activity by 2- to 4-fold (Nicholas et al. 1976).

Since pea leaf asparaginase was affected by energy fluence rates, the involvement of light in the increase of asparaginase activity was examined in more detail with the addition of DCMU or atrazine (inhibitors of non-cyclic electron transport, Trebst 1980). The increase of asparaginase activity in *Chlamydomonas* in the light was inhibited with the addition of 2 μM DCMU, and Paul and Cooksey (1981a) suggested that photosynthesis was required for the increase in asparaginase activity. However, these workers did not demonstrate that the light reactions of photosynthesis were inhibited with the addition of DCMU.

The addition of 20 μM DCMU or atrazine to half expanded pea leaves reduced photosynthetic activity, estimated by O_2 evolution from leaf pieces or *in vivo* chlorophyll *a* fluorescence (figures 19, 20 22), and abolished any light dependant increase in asparaginase activity (figure 22). The absence of a light stimulated increase in asparaginase activity in the presence of DCMU was not reversed by the addition of 10 or 20 mM

sucrose, and indicates that a carbohydrate source could not replace a product of the light reaction. This may suggest that carbohydrate starvation, which could possibly result from reduced photosynthetic electron transport, does not inhibit the increase of asparaginase activity. The addition of sucrose increased extractable asparaginase activity in the absence of DCMU (table 11) and it is possible that sucrose stabilized asparaginase during extraction, although this was not examined. Overall, the above results suggest that photosynthetic electron transport is required for the increase in asparaginase activity.

The addition of products of the light reaction (e.g. reduced ferredoxin, NADPH, ATP), and related compounds (NADH, GSH) to asparaginase preparations (table 12) suggest that the effects of light on asparaginase activity are not through direct modulation of activity, but rather that the effect of light on asparaginase activity is indirect. It is possible that reduced compounds (reduced ferredoxin, NADPH, or NADH) may help stabilize asparaginase (in the presence of asparagine) *in vivo* as they do *in vitro*, and inhibition of photosynthesis limits the supply of these compounds. However, this would not explain the requirement of light for the increase in activity. Another possibility is that the inhibition of photosynthesis reduces the energy charge of the cell which would affect many cellular processes including protein synthesis (DCMU has been reported to inhibit chloroplast protein synthesis, Ellis 1977, and chloroplast DNA biosynthesis, Mills and Baumgartner 1983) which appears to be required for the increase of asparaginase activity (section III.12, figure 38, table 21). Recently it has been shown that the uptake of Ca^{2+} into protoplasts (Heimann et al. 1986) and chloroplasts (Kriemer et al. 1985, Surek et al. 1986) is DCMU-sensitive, and the alteration of,

Ca^{2+} levels within the cell due to DCMU treatment would affect several cellular processes directly or indirectly, possibly including asparaginase activity.

Even though it appears that photosynthetic electron transport is required for the increase in asparaginase activity in half expanded pea leaves, the conclusion should be considered with caution. Several workers have examined the involvement of light in the induction of NR and their conclusions have differed. This is in part due to different plant genera examined, or differences in experimental design, including plant age. Photoinduction of NR in greening rice seedlings (Sawhney and Naik 1972), or greening pea apices (Sihag et al. 1979) was inhibited in the presence of DCMU or atrazine and it was proposed that light was required for NR induction. However, the requirement of light for the induction of NR is still questioned for several reasons:

- 1) the addition of sucrose (40 mM) to greening pea apices overcame the inhibition of NR photoinduction by DCMU, and actually stimulated NR activity 2-fold (in the presence of DCMU) compared to treatments without DCMU or sucrose (Sihag et al. 1979).

- 2) In green leaves the addition of DCMU had no effect on NR activity (Beevers and Hageman 1980, Srivastava 1980, Duke and Duke 1984).

- 3) NR was induced in seedlings darkened for 3 days (with high levels of polyribosomes), while seedlings darkened for 10 days (with low levels of polyribosomes) required light for the induction of NR, suggesting that light was necessary for the development of an active protein-synthesizing apparatus rather than for NR induction *per se* (Travis and Key 1971).

- 4) experiments treating soybean seedlings with norflurazon (an

inhibitor of carotenoid synthesis that impairs chloroplast development) indicated that functional chloroplasts were not required for NR+S induction (i.e. NR induced in the presence of substrate, Kakefuda et al. 1983).

5) Light may regulate the availability of NO_3^- supply, thereby influencing NR activity indirectly (Beevers and Hageman 1980, Quail 1983).

In an effort to consolidate several of these results Duke and Duke (1984) suggested that plastid development and function would only be required for NR and other enzymes with short half-lives after cotyledonary reserves are depleted, or when carbohydrate skeletons are in short supply. Therefore any enzymes that turn over rapidly would depend on current photosynthate for continued synthesis. Since the light dependency of asparaginase activity was examined in developing leaves of a mature plant, it appears probable that current photosynthate would be required for increased activity (e.g. for the synthesis of asparaginase) since reserve carbohydrate might be in short supply. However, in the case of asparaginase, product(s) of photosynthetic electron transport would also be required to stabilize existing asparaginase activity. There is a consensus that light and NO_3^- are required for maximal NR levels (Beevers and Hageman 1980, Srivastava 1980, Guerrero et al. 1981, Duke and Duke 1984) and it appears that light is also required for maximal asparaginase activity.

With higher concentrations of DCMU or atrazine, levels of asparaginase decreased during treatment to below the initial level of asparaginase activity, (i.e. at the end of the dark period). A decrease in

asparaginase, below the activity assayed at the end of the normal dark period, was also observed when plants were maintained in extended darkness for a further 4 to 6 hours (figure 15). These results suggest that, in the absence of photosynthesis, asparaginase is inactivated or that the enzyme is not stable. A analogous conclusion for NR was arrived at by Lillo (1983, 1984b) who observed that NR activity in barley decreased in the dark over a 2 to 4 hour period. Lillo (1983) suggested that there were differential stabilities associated with NR during the photoperiod since NR was less stable at the end of the light period NR than at the end of the dark period. Mixing of the stable and unstable extracts resulted in an activity similar to the mean value and it was concluded that NR degrading enzymes, such as those found in maize (e.g. Wallace 1977) did not play a role in modification of NR in barley. It was proposed that changes in molecular weight (i.e. assembly of subunits) could account for the 'rapid' changes in NR activity, however no data or mechanism were presented to support this view (Lillo 1983).

Differential stabilities of asparaginase may also exist, since asparaginase appeared less stable (as indicated by a greater decrease of activity) at the end of the light period than at the end of the dark period both *in vitro* (figure 4) and *in vivo* (figure 15), and the addition of chymotrypsinogen, an endopeptidase, only reduced asparaginase activity when obtained at the end of the dark period (figure 34). However, the occurrence of differential stabilities of asparaginase activity was not critically examined. No evidence for the presence of a soluble factor(s) was found (by coharvesting, or mixing extracts obtained at the end of the dark or light periods, table 19) that could affect asparaginase activity, nor was there any indication that asparaginase (a dimer, Lea et al. 1978)

underwent a change in relative molecular mass during the photoperiod (figure 35). It is possible however, that the monomer of asparaginase is inactive and would not be detected after S300 chromatography.

When extracts, obtained during the light period, were subject to S300 gel permeation chromatography an increase in the total activity of the preparation was noted (this was not observed for extracts obtained at the end of the dark period, section III.10) suggesting that a soluble inhibitor of asparaginase activity may be present during the light period. The reason for the discrepancy between the coharvesting, and S300 experiments, is not known. An analogous situation, involving the presence of an inhibitor of enzyme activity in the light, appears to occur with PAL. Increased PAL activity (*de novo* synthesis) takes place at a time when a PAL-'inactivating system' (PAL-IS, the main agent of which is "probably" a proteolytic enzyme) is already present (Hanson and Havir 1981). As a result, the decay of PAL activity takes place as it is being synthesized until a point is reached where synthesis of PAL is equal to its breakdown. A lag period from 0.75 to 2.5 hours is noted before PAL activity increases, and this coincides with a decrease in the synthesis of PAL-IS-mRNA (PAL-IS is unstable or inactivated) and the processing, export and translation of PAL-mRNA (Hanson and Havir 1981). To some extent these results are similar to the behaviour of pea asparaginase.

Mechanisms for regulation of asparaginase activity

Changes in the level of enzyme activity might reflect modulation of one or more of these processes: 1) regulation of activity through low molecular weight compounds, 2) activation/inactivation by

protein-mediated processes, 3) protein degradation, or 4) *de novo* synthesis. Studies on the regulation of NR and PAL activities have reported that all four mechanisms play a role in the regulation of enzyme activity (e.g. for NR Srivastava 1980, and Guerrero et al. 1981, or for PAL see Hanson and Havir 1981), and demonstrate that regulation of enzyme activity involves complex interactions of many processes. Of these processes, only *de novo* mRNA and protein synthesis could be shown to account for the increase in asparaginase activity, however, the other three mechanisms were found to modify asparaginase activity to some extent. These results are summarized below

Asparaginase activity does not appear to be regulated *in vivo* by low molecular weight compounds such as ions, pyridine nucleotides or end products of the catalysed reaction. However, asparaginase was inhibited by high levels of ATP or Zn^{2+} , and in the presence of Ca^{2+} higher activity was observed, but what these responses might mean *in vivo* is not clear. No evidence was obtained for the modulation of asparaginase activity through protein mediated systems such as protein kinase, calmodulin, light effect mediator (LEM/protein modulase), or thioredoxin reductase. However, LEM/protein modulase or thioredoxin reductase may be involved in maintaining the stability asparaginase *in vivo*, since asparaginase activity was stable in the presence of reduced ferredoxin, and after chromatographic removal of these proteins from asparaginase this stabilization was lost. Proteolytic degradation of asparaginase may also occur *in vivo* (possibly accounting for the decrease in darkness) since an endopeptidase reduced asparaginase activity when the enzyme was obtained during the light period (figure 34); the decrease was not observed in asparaginase activity obtained at the end of the dark period. However,

additions of proteolytic inhibitors (PMSF, iodoacetate) had no effect on the decrease of asparaginase activity *in vitro* (table 18). In general, no evidence was found (based on coharvesting experiments, table 19) that suggests the involvement of soluble factors in the regulation of asparaginase activity.

Because the light reactions are strongly involved in controlling asparaginase activity, it seemed possible that asparaginase might be localized in the chloroplast. Chloroplasts were rapidly isolated (using the method of Mills and Joy 1980) from half expanded pea leaves during the light period and assayed for activity and no asparaginase activity was detected (data not shown). Asparaginase also does not appear to be modified by the changes in pH or Mg^{2+} (section III. 3) that have been demonstrated to occur in the chloroplast on illumination (Portis et al. 1976). This confirms the earlier report that asparaginase is a cytosolic enzyme (Ireland and Joy 1983b).

To date, only 2 or possibly 3 cytosolic enzymes are known to be dependant on non-cyclic (DCMU-sensitive) electron transport flow for increased or decreased activity. Phosphoenol pyruvate carboxylase (PEP carboxylase, Anderson 1986) and possibly NR, as discussed above, require photosynthetic electron transport for increased activity; glucose 6 phosphate dehydrogenase (G6PD, cytosolic isoform) activity is inactivated as a result of photosynthetic electron transport (Anderson 1986). The present study indicates that asparaginase should be added to this list. Activation of phenylalanine ammonia-lyase (PAL) by light is also DCMU sensitive (Camm and Towers 1973, Hanson and Havir 1980), however two isoforms of PAL are detected in the chloroplast, and one is regulated by thioredoxin (Nishizawa et al. 1979). The decrease in G6PD activity in the

light has been shown to be regulated by LEM (Anderson 1986) or thioredoxin (Buchanan 1980) and thioredoxin_m has been detected in the cytosol (Jacquot 1984) which could possibly account for the sensitivity of G6PD to DCMU. However, mechanisms that control cytosolic enzyme activities by photosynthetic electron transport are currently not understood (Anderson 1986).

Another mechanism that might regulate enzyme activity *in vivo* is shown by chloroplastic G6PD and involves reversible association-disassociation of G6PD with the thylakoid membrane. This was proposed by Ben-Bassett and Anderson (1981) since they observed that G6PD activity was higher when associated with the thylakoid membrane. In their studies, divalent cations promoted association, while anions enhanced dissociation, of G6PD with the membrane. A similar type of mechanism, i.e. altering the location of enzymes from the soluble to particulate phase, has also been demonstrated for several glycolytic enzymes in animal tissues (e.g. Knull 1978, Masters 1985).

The possibility that asparaginase was partitioned between the soluble and particulate phase during the photoperiod was examined. Since activity was routinely monitored in soluble fractions, changes in asparaginase activity associated with the particulate fraction of the extract would not normally be detected. Therefore, the problem was approached by considering whether or not association with a discarded particulate fraction might reduce asparaginase activity detected in the soluble fraction (at the end of the dark period). The addition of Triton X 114 (a nonionic detergent used to solubilize membrane proteins preserving their structure, Hearing et al. 1976) or PO_4^{3-} (found to be effective in releasing

G6PD from membranes) had no effect on particulate asparaginase activity (table 20) which suggested that asparaginase (obtained at the end of the dark period) was not associated with a particulate fraction. Other experiments also indicated that asparaginase does not appear to be associated with a particulate phase during the photoperiod. Treatment of the particulate fraction from preparations obtained at the end of the dark period or after 6 hours in the light with Triton X114 (0.025 to 0.2%) or extraction of asparaginase (both at the end of the dark or during the light period) in 0.1% Triton X114 had no effect on asparaginase activity (data not shown).

The increase in asparaginase activity does not appear to be controlled by phytochrome. When plants were given either a 5 minute exposure of red light (table 17) or a 10 minute exposure to regular light (data not shown) and these treatments were followed with a 4 hour dark period, no increase in asparaginase activity was observed. Exposure of etiolated seedlings to 5 minutes of red light has lead to the induction of NR and PAL, and this response was reversed when far red light immediately followed the red treatment (Lamb and Lawton 1983). The exposure of etiolated seedlings to 2 to 3 hours of continuous far red light (the high irradiance response, HIR) also resulted in an increase of NR activity, yet NR in de-etiolated tissues was not affected by the HIR treatment (Duke and Duke 1984). Interestingly enough, NR (Lillo and Henriksen 1984) or PAL (Creasy 1968) activities were also not affected in green leaves on exposure to red light, and it was proposed that different mechanisms may be involved in the increase of NR in etiolated seedlings or green leaves (Lillo and Henriksen 1984). A similar conclusion was reached by Kloppstech (1985) who noted that mRNA levels in green leaves, coding for Rubisco and the light-harvesting

chlorophyll *a/b* proteins, varied in a circadian manner and were not regulated by phytochrome. However, the mRNA's coding for these proteins in etiolated seedlings are under phytochrome control (Harpster and Apel 1985, Kloppstech 1985, Tobin and Silverthorne 1985). These differences may be explained by the fact that 'multiple switches' may regulate phytochrome control of gene expression, rather than a single 'master switch', as has been demonstrated for phytochrome induction of PAL (Lamb and Lawton 1983). Phytochrome control of asparaginase in etiolated seedlings was not examined because asparaginase activity was very low in this tissue (and during a 6 hour exposure to light, data not shown), as it was in 8 day old plants (table 1).

Experiments employing inhibitors of RNA and protein synthesis have indicated that *de novo* synthesis of protein is required for increased NR (Beever and Hageman 1980, Duke and Duke 1984) or PAL (Lamb and Lawton 1983) activities. A similar approach was carried out in the present study to examine whether the light response had any effect at the transcriptional or translational level with respect to asparaginase activity. Short periods of time for feeding (3.5 hours) were used to minimize secondary effects that arise from the use of inhibitors (Galling 1983).

Cycloheximide, an inhibitor of eukaryotic translational processes (80S ribosomes, acts on chain elongation preventing movement of ribosomes along mRNA, Gale et al. 1984), has been used by many workers to demonstrate the involvement of cytosolic protein synthesis for several enzymes (e.g. Galling 1983, Lamb and Lawton 1983). Feeding cycloheximide to half expanded pea leaves in the light for 3.5 hours had a dramatic effect on asparaginase activity at concentrations above 1.0

µg/ml (figure 38, table 21). At these concentrations, asparaginase activity decreased below the initial value, at the beginning of the feeding (i.e. the value obtained at the end of the dark period). This result was similar to those obtained for asparaginase activity when treated with high concentrations of DCMU, atrazine, or maintained in extended dark periods as discussed earlier.

Cycloheximide is known to affect many cellular processes. For example at 1.0 µg/ml, respiration was stimulated and Cl^- uptake inhibited (MacDonald and Ellis 1969) and NH_3 was reported to accumulate to toxic levels (Bradford and Fletcher 1982). At higher concentrations of cycloheximide K^+ uptake was inhibited (Reilly et al. 1970), and it has been suggested that cycloheximide acts as a glutamine analogue (Jones 1977). Other studies indicated that cycloheximide at 2.0 µg/ml inhibited DNA and RNA synthesis, inhibited the accumulation of stable RNA, stimulated the breakdown of nucleic acids in general, and decreased cellular ATP levels (McMahon 1975). It was therefore proposed that cycloheximide was not a reliable inhibitor of protein synthesis since secondary effects arising from cycloheximide could not be distinguished from the direct effects of cycloheximide on protein synthesis (McMahon 1975). More recently Galling (1983) has suggested that the inhibition of protein synthesis by cycloheximide is a secondary effect. Even though no increase in the levels of NH_3 were observed at 5 µg/ml cycloheximide in the present study, the data for the inhibition of the light-dependant increase of asparaginase by cycloheximide should be considered with some caution.

The addition of puromycin (an analogue of the aminoacyl-adenosine end of aminoacyl-tRNA, takes part in 50S and 60S ribosomal peptidyl

transferase reactions and promotes polyribosome breakdown, Gale 1981) to pea leaves stimulated asparaginase activity at low concentrations (<50 µg/ml) and only marginally inhibited ^{14}C -leucine uptake into ethanol soluble precipitates. At higher concentrations (200 µg/ml), puromycin inhibited the increase of asparaginase in the light by 58% (approximating initial values of asparaginase activity at the end of the dark period, figure 38) and only inhibited ^{14}C -leucine incorporation by 12%. Secondary effects related to puromycin use include nonspecific impairment of the 'nucleo-cytoplasmic system' (Galling 1983), and at 50 µg/ml increased NH_3 levels have been reported (Bradford and Fletcher 1982). However, at 200 µg/ml puromycin, no increase in NH_3 levels were observed in the present study, nor did this concentration of puromycin have an effect on photosynthetic activity (section III.12). These results suggest that translational processes are required for the increase of asparaginase activity in the light. This was also supported by the data obtained with emetine (figure 38, table 21), a specific inhibitor of eukaryotic translation (affects 40S ribosomal subunits, Gale 1981).

The addition of low concentrations (5 to 10 µg/ml) of actinomycin D (an antibiotic that inhibits transcription by intercalating DNA thereby reducing the capacity of DNA to act as a template for RNA synthesis in both prokaryotic and eukaryotic cells, Gale et al. 1981) suggested that mRNA synthesis was required for increased asparaginase activity in the light. Actinomycin D has been reported to have several secondary effects in leukemic leukocytes including inhibition of respiration, and glycolysis and decreasing ATP levels of cells (Laszlo et al. 1966), but in cowpea hypocotyls, actinomycin D (50 µg/ml) stimulated respiration (Johnson et

a.l 1976). Inhibition of asparaginase activity was also noted with the addition of cordycepin, an inhibitor of eucaryotic mRNA synthesis (inhibits polyadenylation of mRNA, since it is incorporated into the 3' end of the growing RNA chain and blocks elongation, Gale 1981), at low concentrations (10 to 20 $\mu\text{g/ml}$) with a corresponding decrease in ^{14}C -leucine incorporation. In animal systems, cordycepin has been found to inhibit RNA efflux from isolated nuclei, most probably due to the requirement of polyadenylated-RNA to interact with a transport system (Agutter and McCaldin 1979), however in plants no side effects have been reported with cordycepin treatments (at 200 $\mu\text{g/ml}$, Aspart et al. 1979). The possibility that RNA export from nuclei is inhibited by cordycepin appears likely. The inhibition of the increase in asparaginase activity with cordycepin again suggested that newly synthesized mRNA was required for the increase of asparaginase activity in the light. Inhibition (Sihag et al. 1979) and stimulation of NR activity have been reported in the presence of cordycepin and actinomycin D (Duke and Duke 1984) indicating that preformed NR-mRNA was present in some of the tissues examined.

Asparaginase activity was stimulated on the addition of chloramphenicol (the D-threo isomer, an inhibitor of prokaryotic translation inhibiting the peptidyl transferase reaction on 50S ribosomes, Gale 1981) to half expanded pea leaves, even at concentrations of 100 $\mu\text{g/ml}$. At high concentrations (> 300 $\mu\text{g/ml}$) chloramphenicol has been found to inhibit ion accumulation, or uncouple oxidative phosphorylation (Hanson and Hodges 1963) and reduce respiration (Boulter 1970, Johnson et al. 1976). However, concentrations well below those reported to have secondary effects were used in the present study. A stimulatory effect on NR activity by chloramphenicol was reported for rice (at 500 $\mu\text{g/ml}$, Shen

1972, Sawheny and Naik 1973), maize ('low' concentrations, 1.6 mg/ml, Schrader et al. 1967) and pea (Sihag et al. 1979) seedlings. However, at higher concentrations chloramphenicol inhibited NR induction in maize (3 to 6 mg/ml, Schrader et al. 1967), or beans (Srivastava 1980). The positive effect on asparaginase activity, resulting from inhibition of chloroplast protein synthesis, could possibly be manifested in two ways: 1) the reduction of plastid protein synthesis could allow more ATP to be available for other cellular processes, or 2) an inhibitor (the synthesis of which is inhibited by chloramphenicol) of asparaginase could be synthesized in the plastid during the light. Similar suggestions were proposed for the stimulation of NR (Srivastava 1980, Duke and Duke 1984).

These results indicate the requirement for mRNA and protein synthesis for the increase in asparaginase activity and suggest that light may enhance the capacity for cytosolic protein synthesis.

Other *in vivo* factors influencing asparaginase

Data have been presented and discussed that suggest that *de novo* protein synthesis may account for the increase in asparaginase activity in the light. However, a series of experiments were performed that indicate that light is not the only signal that modifies asparaginase activity. No increase in asparaginase activity could be demonstrated when half-expanded leaves obtained from hydroponic plants were fed in the light, even though leaves left on the plant over the same interval of time displayed an increase in asparaginase activity (section 1.10, table 8). Xylem sap analysis indicated that the nitrogen content of the transpiration streams were similar between soil and hydroponically grown pea plants (table 6).

In an effort to reduce the level of xylem or leaf constituents that may have accounted for the differences noted in asparaginase activity, shoots from soil and hydroponically grown plants were supplied with water for 24 hours before a feeding experiment was performed (table 8). No increase in asparaginase activity was observed in half expanded leaves from either soil or hydroponically grown plants following this treatment. Feeding of soil xylem sap to hydroponic leaves did not increase their asparaginase activity, and feeding hydroponic xylem sap to half expanded soil leaves, only marginally reduced the increase of activity asparaginase activity (compared to control treatment, table 7). The lack of increase in hydroponic leaves does not appear to be due to a reduced capacity for protein synthesis in these leaves since ^{14}C -leucine incorporation was similar in soil or hydroponically grown leaves (section III.12).

The inactivation of asparaginase *in vivo* was noted when half expanded leaves, obtained from soil grown plants 6 hours after the onset of the light period, were detached and placed into feeding solutions, maintained in the light. Asparaginase activity decreased in these leaves during the first hour of feeding in the light (section I.11, table 10) and activity remained at a low level for 6 hours in the light, the longest time examined. A similar decrease in asparaginase activity was also observed when leaves (from soil grown plants) were excised after 14 hours of illumination and fed in the light (data not presented). It is interesting to note that products of photosynthesis (and the capacity for protein synthesis) would still be present for the maintenance and renewal of asparaginase activity in these leaves since they are fed in the light, however, asparaginase activity decreased. It is possible that the content of the transport streams may affect the light-dependant increase and

maintainance of asparaginase activity. However, these experiments did not identify the prime cause for the differences observed between detached leaves of soil and hydroponically grown plants during feeding experiments; the reason for decreased asparaginase activity in leaves, detached during the light period, is not known.

Asparaginase and NH_3 metabolism

One of the major sources of nitrogen available to young developing leaves results from the hydrolysis of asparagine, by asparaginase, producing NH_3 and aspartate. Several other processes are also known to liberate NH_3 in leaf tissues for example: glycine-serine conversion results in photorespiratory NH_3 release, the combined actions of NR and NiR activities reduce inorganic nitrogen to the level of NH_3 , PAL, threonine dehydratase, other deamidases (glutaminase, and α -amidases) and urease produce NH_3 as a reaction product, and NH_3 is also liberated during protein turnover. It has been suggested that in the light the production of NH_3 by photorespiration exceeds other sources (see below). When leaf homogenates were analyzed for NH_3 , the levels were low and relatively constant throughout the photoperiod (figure 8). It is currently thought that the primary route for NH_3 assimilation in plant tissues is catalyzed by glutamine synthetase (GS) in conjunction with glutamate synthase (GOGAT), rather than being catalyzed by glutamate dehydrogenase (GDH). This conclusion is based on analysis of the affinities of GS and GDH for NH_3 , and ^{15}N feeding studies which have demonstrated a rapid increase of ^{15}N in the amide group of glutamine

from several ^{15}N sources (Mifflin and Lea 1983).

In the present study GS activity varied in an ultradian manner in developing pea leaves (figure 8) and this observation is similar to that reported for GS in bean or maize leaves (Duke et al. 1978) and sunflower roots (Knight and Weissman 1982). In bean and maize leaves GDH activity varied throughout the photoperiod, however activity decreased in the light period (Duke et al. 1978). In the present study GDH activity also decreased in the light period (figure 9). Glutamine synthetase appears to be an efficient scavenger of cellular NH_3 since the levels of NH_3 were low during the light period, even though GS activity varied over this time period. Walker et al. (1984a) observed that feeding 10 mM NH_3 to leaves resulted in only a slight increase in NH_3 levels within the leaf (although glutamine levels increased) and suggested that the GS-GOGAT cycle has excess capacity to assimilate more NH_3 than that supplied by NO_3^- reduction and photorespiration.

Concentrations of methionine sulfoximine (MSO, an irreversible inhibitor of GS activity, Tate and Meister 1973, Mifflin and Lea 1980), from 1 to 10 mM have been used to demonstrate the involvement of GS in the assimilation of NH_3 derived from photorespiration in C_3 and C_4 leaf tissues (Keys et al. 1978, Frantz et al. 1982, Larsson et al. 1982, Achhireddy et al. 1983, Berger and Fock 1983a,b, 1985, Kumar et al. 1984, Martin et al. 1983). Studies have also indicated that photorespiratory sources of NH_3 are not the only NH_3 sources within leaf tissues, since similar levels of NH_3 (in the presence of MSO) have been

detected under conditions known to support (high O_2) or limit (low O_2) photorespiration (Platt and Anthon 1981, Platt and Rand 1983).

If GS is inhibited with MSO, NH_3 currently being produced in various reactions will accumulate. A more rapid production of NH_3 by asparaginase (and other light modulated enzymes) might be expected after light treatment. The accumulation of NH_3 from these sources would only be detected if the rapid production from photorespiration can also be inhibited, by the use of aminooxyacetate (AOA, an inhibitor of transamination reactions, Givan 1980, also see section I.11). However, an increase of NH_3 in the presence of MSO and AOA was not observed (yet in the presence of MSO, NH_3 levels were high, table 9). A similar result was also reported by Yamaya and Matsumoto (1985) who fed pea shoots MSO and AOA in the light or dark and examined NH_3 levels. They noted that in the presence of MSO and AOA the liberation of NH_3 was just above those of the control treatments for both dark or light treatments (MSO alone resulted in high levels of NH_3). In the present study, the addition of 0.5 to 5 mM MSO, without AOA, to half expanded leaves inhibited GS activity and caused an increase in NH_3 levels, thus it was effective at preventing NH_3 assimilation.

Methionine sulfoximine has been reported to inhibit photosynthesis (measured as net CO_2 fixation; Platt and Anthon 1981, Platt and Rand 1982, Achhireddy et al. 1983, Ikeda et al. 1984, Walker et al. 1984b, Berger and Fock 1983b, 1985) but the mechanism by which this inhibition takes place is not known. Platt and Anthon (1981) and Achhireddy et al.

(1983) proposed that the accumulation of NH_3 , in the presence of MSO, may inhibit photosynthesis since concentrations above 1.0 mM NH_3 are known to be toxic in plants (e.g. Givan 1979). Ikeda et al. (1984), Berger and Fock (1983b) and Walker et al. (1984b) have suggested that factors other than NH_3 accumulation might inhibit photosynthesis in the presence of MSO. It was suggested that depletion of calvin cycle intermediates (inhibition of GS by MSO would lead to reduced cycling of photorespiratory intermediates and decreased reshuttling of carbon skeletons back into the calvin cycle) may reduce photosynthesis. Ikeda et al. (1984) also proposed that increased glycolate levels (again a result of reduced photorespiratory cycling) may reversibly inhibit Rubisco and reduce photosynthesis. Reduced photosynthesis does not appear to be a result of stomatal closure since MSO has no effect on transpiration rates (Platt and Anthon 1981; Walker et al. 1984b).

Increased asparaginase activity is dependant on photosynthetic activity, therefore, it seemed possible that MSO, by inhibiting photosynthesis, may also reduce the light-dependant increase in asparaginase activity. At concentrations as low as 0.5 mM, MSO did inhibit the light dependant increase in asparaginase activity (figure 14). Reduced photosynthesis in the presence of MSO may explain why no increase was observed in asparaginase activity in the light with added MSO.

Several studies have examined the accumulation of NH_3 in the presence or absence of inhibitors of the photorespiratory pathway (AOA, an inhibitor transaminase reactions, isonicotinyl hydrazide, INH, or aminoacetonitrile, AAN, inhibitors of the glycine decarboxylase complex,

Sarojini and Oliver 1985) and in the absence of these inhibitors, levels of NH_3 were reported to be higher than in their presence. Results obtained by using MSO+AOA (Martin et al 1983, Yamaya and Mutsumoto 1985), MSO+INH (Martin et al. 1983, Kumar et al, 1984, Walker et al. 1984), or MSO+AAN (Achhireddy et al. 1983) have been presented as support for the idea that the major source of NH_3 in leaves (an order of magnitude higher than primary assimilation, Keys et al 1978) is from photorespiration. Similar conclusions have also been reported from studies using only MSO (Keys et al. 1978, Platt and Anthon 1981, Larson et al. 1982, Platt and Rand 1982, Berger and Fock 1983a,b, 1985, Ikeda et al. 1984).

The inhibition of the increase of asparaginase (and photosynthetic) activity *in vivo* in the presence of MSO, indicates that results estimating the levels of NH_3 in leaves using MSO are not representative of NH_3 production from all sources. Any reactions liberating NH_3 that are directly dependant on light for activity, such as asparaginase, NR, NiR, and PAL, may be inhibited in the presence of MSO. Therefore, the experimental design skews the result to measure sources of NH_3 that arise indirectly from light-stimulated reactions, such as photorespiratory NH_3 production, rather than light-dependant reactions. It would be of interest to examine the effect of MSO on the *in vivo* activities of other NH_3 producing enzymes that increase activity in the presence of light such as NR, NiR and PAL.

The addition of MSO and AOA have also been used to demonstrate that the flow of nitrogen from asparagine in half expanded pea leaves is

predominantly through asparagine transamination, rather than through asparagine deamidation (Ireland and Joy 1981, Ta et al. 1984a,b; see Introduction: "Support for asparagine metabolism in leaves with ^{15}N experiments"). In view of the inhibition of asparaginase during MSO feeding experiments, these results are not representative of asparagine metabolism *in vivo* and it is likely that asparaginase plays a more important role in supplying nitrogen in developing leaves than was previously proposed.

Why does asparaginase activity vary diurnally?

High levels of NH_3 (above 1 mM) are dangerous to plant cells. Under these conditions, uncoupling of oxidative and photophosphorylation may occur (Givan 1979), reducing cellular levels of ATP at a time when ATP is needed for GS activity. A shift of metabolism from predominantly sugar synthesis to increased amino acid synthesis has also been observed (e.g. Platt et al. 1977, Larsen et al. 1981, Marques et al. 1983, Kanazawa et al. 1983, Walker et al. 1984a) under high NH_3 conditions along with the stimulation of $^{14}\text{CO}_2$ fixation (Woo and Calvin 1980) and O_2 evolution (Givan 1979). Flux through the glycolytic pathway usually is low during the light period under nonstressed conditions (Turner and Turner 1980). However, elevated levels of NH_3 results in increased glycolytic activity during the light period (e.g. Kanazawa et al. 1983) and increased activities of pyruvate kinase (Platt et al. 1977, Larson et al. 1981, Kanazawa et al. 1983), and phosphoenolpyruvate carboxylase (Larson et al. 1981, Kanazawa et al. 1983) coincide with the stimulated glycolytic flux. High levels of NH_3 have also been reported to stimulate chloroplast

FBPase, NADP-malate dehydrogenase (Rosa and Whatley 1981), and NADP-glyceraldehyde dehydrogenase (Mohamed and Gnanam 1979) activities. Thus NH_3 may cause a wide range of perturbations of normal metabolism. In addition, above 10 mM NH_3 , ultrastructural damage of the chloroplast due to swelling results (Puritch and Barker 1967).

Since NH_3 is toxic to cells, reactions giving rise to NH_3 as one of the products might be expected to be under some form of regulation (however crude it might be), so that NH_3 is produced when it can be assimilated and further metabolized most efficiently. Assimilation of NH_3 by GS and the subsequent metabolism of glutamine are dependant on products of the light reactions of photosynthesis (Wallsgrave et al. 1983). Jordan and Givan (1979) have suggested that the principal (but not only) source of ATP for GS activity was supplied by photophosphorylation since glutamine synthesis was inhibited by DCMU, and Ito et al. (1978) have demonstrated that ^{15}N -amide glutamine is utilized very slowly in the dark. Photorespiratory NH_3 release is indirectly regulated by light through the activity of Rubisco (known to be light regulated, Buchanan 1980) and increased NH_3 levels, assumed to be derived from photoprespiration, have been reported several times in the presence of MSO in the light (Platt and Rand 1982, Achhireddy et al. 1983, Walker et al. 1984b, Yamaya and Matsumoto 1985). The activities of asparaginase, NR, NIR, and PAL are DCMU sensitive and dependant upon light to some extent for activity.

It is proposed that the light dependant increase in asparaginase activity (as for other enzymes that produce NH_3) is a strategy that

ensures that free NH_3 is produced mainly during the light period when assimilation of NH_3 and subsequent metabolism of glutamine is optimal. During darkness, asparaginase activity (and other enzymes producing NH_3) progressively decreases, reducing potential NH_3 production. Since high levels of free NH_3 in darkness are potentially dangerous to a cell, the 'down-regulation' of asparaginase in the dark is an important aspect of asparagine metabolism.

Conclusions

High asparaginase activity was found in young developing pea leaves obtained from pea plants; many factors were found to influence this activity. A complex interaction between asparaginase and ions was noted, but none were found to play a role in regulation of asparaginase activity. Greater activity was found when asparaginase was extracted and maintained in the presence of Ca^{2+} , but, increased activity was not observed if Ca^{2+} was added to asparaginase preparations. No mechanism was found that could explain this result.

Asparaginase activity varied diurnally in developing pea leaves; an increase in activity occurred during the light period. Increased activity was also observed in detached leaves obtained from soil-grown plants fed water or several other amino acids for a 2.5 to 4 hour period in the light. However, a light-induced increase in asparaginase activity could not be demonstrated in detached leaves from hydroponically grown plants. Asparaginase activity was also found to decrease rapidly in detached leaves from soil grown plants when obtained and maintained in the light. At present there is no obvious mechanism to explain these

results. Aside from diurnal variation of activity, asparaginase activity also decreased with individual leaf age and maximal activity in each leaf increased with plant age. These results suggest that several factors are involved in the regulation of asparaginase activity.

Deamidation of asparagine in developing leaves, producing NH_3 and aspartate, is important in supplying nitrogen for amino acid and protein synthesis. Increased levels of NH_3 are toxic to the cell and therefore the activity of asparaginase must be regulated so that liberation of NH_3 mainly occurs during the light period when assimilation is efficient. Asparaginase activity was found to vary diurnally in developing pea leaves. During the dark period asparaginase activity was low, and it continued to decrease over the first hour of illumination. After the initial decrease in activity during the light period, asparaginase activity in half expanded leaves increased rapidly, remaining active for the rest of the light period. An hour lag preceded the inactivation of asparaginase in the dark and asparaginase decreased to a low level of activity for the rest of the dark period. Dark deactivation of asparaginase is also an essential part of the regulation of asparaginase activity; this aspect of regulation was not investigated, but is consistent with protein breakdown.

Asparaginase activity was greater with increased energy fluence rates, and photosynthetic electron transport was required for the increase of asparaginase activity. Products of photosynthetic electron transport, reduced ferredoxin and NADPH, were found to stabilize asparaginase activity in the presence of asparagine, while ATP inhibited activity. To date only two other cytosolic enzymes have been reported to

be affected by DCMU; the mechanism of this interaction is unknown. Of the four general mechanisms regulating enzyme activity (low molecular weight compounds, protein-mediated activation/inactivation, protein degradation, and protein synthesis), *de novo* synthesis of mRNA and protein are required for increased asparaginase activity during the light (activity stabilized in the light, through a protein-mediated process involving LEM-protein modulase/thioredoxin-ferredoxin reductase), proteolysis might account for the decrease in darkness.

In 1977 Smith et al. presented a list of 39 light regulated enzymes that required from minutes to more than 24 hours for increased activity, 6 years later Lamb and Lawton (1983) updated the list to include 61 light regulated enzymes: now asparaginase also needs to be added.

Literature Cited

- Abrol, Y.P., S.K. Sawhney, and M.S. Naik. 1983. Light and dark assimilation of nitrate in plants. *Plant, Cell, Environ.* 6: 595-599.
- Achhireddy, N.R., D.R. Vann, J.S. Fletcher, and L. Beevers. 1983. The influence of methionine sulfoximine on photosynthesis and nitrogen metabolism in excised pepper (*Capsicum annuum* L.) leaves. *Plant Sci. Lett.* 32: 73-78.
- Agutter, P.S. and B. McCaldin. 1979. Inhibition of ribonucleic acid efflux from isolated SV40-3T3 cell nuclei by 3'-deoxyadenosine (Cordycepin). *Biochem. J.* 180: 371-378.
- Akazawa, T. 1979. Ribulose-1,5-biphosphate carboxylase. Pp. 208-229 /n *Encyclopedia of plant physiology* (M. Gibbs and E. Latzko, Eds.). Vol. 6. Berlin, Springer-Verlag.
- Anderson, L.E. and M. Avron. 1976. Light modulation of enzyme activity in chloroplasts. *Plant Physiol.* 57: 209-213.
- Anderson, L.E. 1979. Interaction between photochemistry and activity of enzymes. Pp. 271-281 /n *Encyclopedia of plant physiology* (M. Gibbs and E. Latzko, Eds.). Vol. 6. Berlin, Springer-Verlag.
- Anderson, L.E. 1986. Light-dark modulation of enzyme activity in plants. *Adv. Bot. Res.* 12: 3-46.
- Anderson, L.E., A.R. Ashton, D. Ben-Bassat, A.H. Mohamed, and R. Scheibe. 1980. Modulation of chloroplast enzyme activity: the light-effect-mediator (LEM) system. *What's new in Plant Physiol.* 11: 37-40.
- Arima, K., T. Sakamoto, C. Aaraki, and G. Tamura. 1972. Production of extracellular L-asparaginases by microorganisms. *Agric. Biol. Chem.* 36: 356-361.

- Aspart, L., R. Cooke, and M. Delseny. 1979. Stability of polyadenylic and polyadenylated ribonucleic acids in radish (*Raphanus sativus*) seedlings. *Biochem. Biophys. Acta* 564: 43-54.
- Atkins, C.A., J.S. Pate, and D.L. McNeill. 1980. Phloem loading and metabolism of xylem-borne amino compounds in fruiting shoots of a legume. *J. Expt. Bot.* 31: 1509-1570.
- Atkins, C.A., J.S. Pate, and P.J. Sharkey. 1975. Asparagine metabolism - Key to the nitrogen nutrition of developing legume seeds. *Plant Physiol.* 56: 807-812.
- Atkins, C.A., J.S. Pate, M.B. Peoples, and K.W. Joy. 1983. Amino acid transport and metabolism in relation to the nitrogen economy of a legume leaf. *Plant Physiol.* 71: 841-848.
- Baker, N.R. and M. Bradbury. 1981. Possible applications of chlorophyll fluorescence techniques for studying photosynthesis *in vivo*. Pp. 355-373 *in* Plants and the daylight spectrum (H. Smith, Ed.). New York, Academic Press.
- Bauer, A., A.A. Urquhart, and K.W. Joy. 1977a. Amino acid metabolism of pea leaves, diurnal changes and amino acid synthesis from ^{15}N -nitrate. *Plant Physiol.* 59: 915-919.
- Bauer, A., K.W. Joy, and A.A. Urquhart. 1977b. Amino acid metabolism of pea leaves, labeling studies on utilization of amides. *Plant Physiol.* 59: 920-924.
- Beevers, L. and R.H. Hageman. 1980. Nitrate and nitrite reduction. Pp. 116-168 *in* The biochemistry of plants (B.J. Mifflin, Ed.). Vol. 5. New York, Academic Press.
- Beevers, L. and R. Storey. 1976. Glutamate synthetase in developing cotyledons of *Pisum sativum*. *Plant Physiol.* 57: 862-866.

- Ben-Bassat, D. and L.E. Anderson. 1981. Light-induced release of bound glucose-6-phosphate dehydrogenase to the stroma in pea chloroplasts. *Plant Physiol.* 68: 279-283.
- Bennett, J. 1983. Regulation of photosynthesis by chloroplast protein phosphorylation. *Phil. Trans. R. Soc. Lond. B* 302: 113-125.
- Berger, M.G. and H.P. Fock. 1983a. ^{15}N and inhibitor studies on the photorespiratory nitrogen cycle in maize leaves. *Photosynth. Res.* 4: 3-7.
- Berger, M.G. and H.P. Fock. 1983b. Effects of methionine sulfoximine and glycine on nitrogen metabolism of maize leaves in the light. *Aust. J. Plant Physiol.* 10: 187-194.
- Berger, M.G. and H.P. Fock. 1985. Comparative studies on the photorespiratory nitrogen metabolism in wheat and maize leaves. *J. Plant Physiol.* 119: 257-267.
- Blowers, D.P., A. Hetherington, and A. Trewavas. 1985. Isolation of plasma-membrane-bound calcium/calmodulin-regulated protein kinase from pea using Western blotting. *Planta* 166: 208-215.
- Bolleter, W.T., C.J. Bushman, and P.W. Tidwell. 1961. Spectrophotometric determination of ammonia as indophenol. *Anal. Chem.* 33: 592-594.
- Boulter, D. 1970. Protein synthesis in plants. *Ann. Rev. Plant Physiol.* 21: 91-114.
- Bradford, J.A. and J.S. Fletcher. 1982. Influence of protein synthesis on NO_3^- reduction, NH_4^+ accumulation, and amide synthesis in suspension cultures of Paul's scarlet rose. *Plant Physiol.* 69: 63-66.
- Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72: 248-254.

- Broom, J.D. 1961. Evidence that the L-asparaginase activity of guinea pig serum is responsible for its antilymphoma effects. *Nature* 191: 1114-1115.
- Bryan, J.K. 1980. Synthesis of the aspartate family and branched-chain amino acids. Pp. 403-452 /n *The biochemistry of plants*. (B.J. Mifflin, Ed.). Vol. 5. New York, Academic Press.
- Buchanan, B.B. 1980. Role of light in the regulation of chloroplast enzymes. *Ann. Rev. Plant Physiol.* 31: 341-374.
- Burris, R.H. 1972. Nitrogen fixation - assay methods and techniques. *Methods in Enzymology* XXIV: 415-420.
- Camm, E.L. and G.H.N. Towers. 1973. Phenylalanine ammonia lyase. *Phytochem.* 12: 961-973.
- Canvin, D.T., J.A. Berry, M.R. Badger, H. Fock, and C.B. Osmond. 1980. Oxygen exchange in leaves in the light. *Plant Physiol.* 66: 302-307.
- Carr, D.J. and J.S. Pate. 1967. The changing interrelationships of organs during ageing. *Symp. Soc. for Exper. Biol.* 21: 559-600.
- Castric, P.A., K.J.F. Farnden, and E.E. Conn. 1972. Cyanide metabolism in higher plants. V. The formation of asparagine from β -cyanoalanine. *Arch. Biochem. Biophys.* 152: 62-69.
- Chang, K.S. and K.J.F. Farnden. 1981. Purification and properties of asparaginase from *Lupinus arboreus* and *Lupinus angustifolius*. *Arch. Biochem. Biophys.* 208: 49-58.
- Charles, S.A. and B. Halliwell. 1980. Action of calcium ions on spinach (*Spinacia oleracea*) chloroplast fructose bisphosphatase and other enzymes of the Calvin cycle. *biochem. J.* 188: 775-779.
- Chaykin, S. 1969. Assay of nicotinamide deamidase, determination of ammonia by the indophenol reaction. *Anal. Biochem.* 31: 375-382.

- Cheuk, W.L. and G. Finne. 1984. Enzymatic determination of urea and ammonia in refrigerated seafood products. *J. Agric. Food Chem.* 32: 14-18.
- Chibnall, A.C. 1939. Protein metabolism in the plant. New Haven, Yale Univ. Press.
- Cincerova, A. 1969. Effect of trophic conditions on asparagine transamination in wheat plants. *Biologia Plantarum*. 11: 139-148.
- Cohen, P. 1983. Control of enzyme activity. New York, Chapman and Hall.
- Conway, E.J. and E. O'Malley. 1942. Microdiffusion methods. Ammonia and urea using buffered absorbents (revised methods for ranges greater than 10 μ g. N). *Biochem.* 36: 655-661.
- Cooney, D.A., H.N. Jayaram, S.G. Swengros, S.C. Alter, and M. Levine. 1980. The metabolism of L-asparagine in *Asparagus officinalis*. *Int. J. Biochem.* 11: 69-83.
- Creasy, L.L. 1968. The increase in phenylalanine ammonia-lyase activity in strawberry leaf disks and its correlation with flavonoid synthesis. *Phytochem.* 7: 441-446.
- Cullimore, J.V. 1981. Glutamine synthetase of *Chlamydomonas*: rapid deactivation. *Planta* 152: 587-591.
- Curthoys, N.P. and O.H. Lowry. 1973. The distribution of glutaminase isoenzymes in the various structures of the nephron in normal, acidotic, and alkalotic rat kidney. *J. Biol. Chem.* 248: 162-168.
- Delieu, T. and D.A. Walker. 1981. Polarographic measurement of photosynthetic oxygen evolution by leaf discs. *New Phytol.* 89: 165-178.

- de Looze, S. and E. Wagner. 1983. *In vitro* and *in vivo* regulation of chloroplast glyceraldehyde-3-phosphate dehydrogenase isozymes from *Chenopodium rubrum*. III. The molecular basis of the aggregation phenomenon: chloroplast glyceraldehyde-3-phosphate dehydrogenase as an ambiquitous enzyme. *Physiol. Plant.* 57: 243-249.
- Demming, B. and H. Gimmler. 1983. Properties of the isolated intact chloroplast at cytoplasmic K^+ concentrations. *Plant Physiol.* 73: 169-174.
- De Ruiter, H. and C. Kolloffel. 1983. Arginine catabolism in the cotyledons of developing and germinating pea seeds. *Plant Physiol.* 73: 525-528.
- De Ruiter, H. and C. Kolloffel. 1982. Activity of enzymes of arginine metabolism in the cotyledons of developing and germinating pea seeds. *Plant Physiol.* 70: 313-315.
- Dewolfs, R., G. Broddin, H. Clysters, and H. Deelstra. 1975. Comparison of two electrodes for the determination of ammonia and nitrogen compounds. *Z. Anal. Chem.* 275: 337-341.
- Dilworth, M.F. and L. Dure, III. 1978. Developmental biochemistry of cotton seed embryogenesis and germination. *Plant Physiol.* 61: 698-702.
- Dougall, D.K. 1974. Evidence for the presence of glutamate synthase in carrot cell cultures. *Biochem. Biophys. res. Commun.* 58: 639-646.
- Drains, C., J.R. Kinghorn, and J.A. Pateman. 1977. Aspartic hydroxamate resistance and asparaginase regulation in the fungus *Aspergillus nidulans*. *J. Gen. Microbiol.* 98: 493-501.

- Dubois, E., M. Grenson, and J.-M. Wiame. 1974. The participation of the anabolic glutamate dehydrogenase in the nitrogen catabolite repression of arginase in *Saccharomyces cerevisiae*. Eur. J. Biochem. 48: 603-616.
- Dubois, E., S. Vissers, M. Grenson, and J.-M. Wiame. 1977. Glutamine and ammonia in nitrogen catabolite repression of *Saccharomyces cerevisiae*. Biochem. Biophys. Res. Commun. 75: 233-239.
- Duke, S.H. and S.O. Duke. 1984. Light control of extractable nitrate reductase activity in higher plants. Physiol. Plant. 62: 485-493.
- Duke, S.H., J.W. Friedrich, L.E. Schrader, and W.L. Koukkari. 1978. Oscillations in the activities of enzymes of nitrate reduction and ammonia assimilation in *Glycine max* and *Zea mays*. Physiol. Plant. 42: 269-276.
- Dunlop, P.C. and R.J. Roon. 1975. L-asparaginase of *Saccharomyces cerevisiae* an extracellular enzyme. J. Bacteriol. 122: 1017-1024.
- Dunlop, P.C., G.M. Meyer, and R.J. Roon. 1980. Nitrogen catabolite repression of asparaginase II in *Saccharomyces cerevisiae*. J. Bacteriol. 183: 422-426.
- Dunlop, P.C., R.J. Roon, and H.L. Even. 1976. Utilization of D-asparagine by *Saccharomyces cerevisiae*. J. Bacteriol. 125: 999-1004.
- Dunlop, P.C., G.M. Meyer, D. Ban, and R.J. Roon. 1978. Characterization of two forms of asparaginase in *Saccharomyces cerevisiae*. J. Biol. Chem. 253: 1297-1304.
- Durden, D.L., A.M. Salazar, and J.A. Distasio. 1983. Kinetic analysis of hepatotoxicity associated with antineoplastic asparaginases. Cancer Res. 43: 1602-1605.

- Edwards, G.E., H. Nakamoto, J.N. Burnell, and M.D. Hatch. 1985. Pyruvate, P_i dikinase and NADP-malate dehydrogenase in C_4 photosynthesis: properties and mechanism of light/dark regulation. *Ann. Rev. Plant Physiol.* 36: 255-286.
- Ellis, R.J. 1977. Protein synthesis by isolated chloroplasts. *Biochem. Biophys. Acta.* 463: 185-215.
- Engel, P.C. 1981. Enzyme kinetics. New York, Chapman and Hall.
- Errera, M. 1949. Liver glutaminases. *J. Biol. Chem.* 178: 483-493.
- Fischer, K.H. and E. Latzko. 1979. Chloroplast ribulose-5-phosphate kinase: light-mediated activation, and detection of both soluble and membrane-associated activity. *Biochem. Biophys. Res. Comm.* 89: 300-306.
- Flowers, T.J. and A. Lauchli. 1983. Sodium versus potassium: substitution and compartmentation. Pp. 652-679 // *Encyclopedia of plant physiology* (A. Lauchli and R.L. Bielski, Eds.). Vol. 15B. Berlin, Springer-Verlag.
- Flugge, U.I., M. Freisl, and H.W. Heldt. 1980. The mechanism of the control of carbon fixation by the pH in the chloroplast stroma. *Planta* 149: 48-51.
- Folin and Wu. 1965. Pp. 1326-1327 // *Hawk's physiological chemistry* (B. Oser, Ed.). 14th ed. New York/London, McGraw-Hill.
- Fowler, M.W., W. Jessup, and G.S. Sarkissian. 1974. Glutamate synthetase type activity in higher plants. *FEBS Letts.* 46: 340-342.
- Frantz, T.A., D.M. Peterson, and R.D. Durbin. 1982. Sources of ammonium in oat leaves treated with tabtoxin or methionine sulfoximine. *Plant Physiol.* 69: 345-348.

- Furuhashi, K. and Y. Takahashi. 1982. Control of glutamate dehydrogenase from green tobacco callus mitochondria by Ca^{2+} and pH. *Plant Cell Physiol.* 23: 179-184.
- Gale, E.F., E. Cundliffe, P.E. Reynolds, M.H. Richmond, and M.J. Waring. 1981. The molecular basis of antibiotic action. London, J. Wiley and Sons.
- Galling, G. 1983. Use (and misuse) of inhibitors in gene expression. Pp 663-677 /in *Encyclopedia of plant physiology* (B. Parthier and D. Boulter, Eds.). Vol. 14B. Berlin, Springer-Verlag.
- Gamborg, O.L. 1965. Transamination in plants - the specificity of an aminotransferase from mung bean. *Can. J. Biochem.* 43: 723-730.
- Gella, F.J. and M.A. Pascual. 1982. Assay of glutaminase activity by colorimetric determination of glutamate. *Anal. Biochem.* 127: 322-325.
- Givan, C.V. 1979. Metabolic detoxification of ammonia in tissues of higher plants. *Phytochem.* 18: 375-382.
- Givan, C.V. 1980. Aminotransferases in higher plants. Pp. 329-357 /in *The biochemistry of plants* (B.J. Mifflin, Ed.). Vol. 5. New York, Academic Press.
- Gomes, M.A.F. and L. Sodek. 1984. Allantoinase and asparaginase activities in maturing fruits of nodulated and non-nodulated soybeans. *Physiol. Plant.* 62: 105-109.
- Goto, K. 1984. Causal relationships among metabolic circadian rhythms in *Lemna*. *Z. Naturforsch.* 39c: 73-84.
- Greenstein, J.P. and C.E. Carter. 1946. The influence of α -keto acids on the desamidation of amino acid amides. *J. Biol. Chem.* 165: 741-742.

- Greenstein, J.P. and V.E. Price. 1949. α -keto acid-activated glutaminase and asparaginase. J. Biol. Chem. 178: 695-705.
- Guerrero, M.G., J.M. Vega, and M. Losada. 1981. The assimilatory nitrate-reducing system and its regulation. Ann. Rev. Plant Physiol. 32: 169-204.
- Gupta, S.C. and L. Beevers. 1985. Regulation of synthesis of nitrite reductase in pea leaves: *in vivo* and *in vitro* studies. Planta 166: 89-95.
- Hampp, R., M. Goller, H. Fullgraf, and I. Everle. 1985. Pyridine and adenine nucleotide status, and pool sizes of a range of metabolites in chloroplasts, mitochondria and the cytosol/vacuole of *Avena* mesophyll protoplasts during dark/light transition: effect of pyridoxal phosphate. Plant Cell Physiol. 26: 99-108.
- Hanson, J.B. and T.K. Hodges. 1963. Uncoupling action of chloramphenicol as a basis for the inhibition of ion accumulation. Nature 200: 1009.
- Hanson, K.R. and E.A. Havir. 1981. Phenylalanine ammonia-lyase. Pp. 577-625 /n The biochemistry of plants (E.E. Conn, Ed.). Vol. 7. New York, Academic Press.
- Harpster, M. and K. Apel. 1985. The light-dependent regulation of gene expression during plastid development in higher plants. Physiol. Plant. 64: 147-152.
- Hearing, V.J., W.G. Klingler, T.M. Ekel, and P.M. Montague. 1976. Molecular weight estimation of triton X-100 solubilized proteins by polyacrylamide gel electrophoresis. Anal. Biochem. 72: 113-122.
- Heber, U. 1974. Metabolite exchange between chloroplasts and cytoplasm. Ann. Rev. Plant Physiol. 25: 393-421.

- Helde, O.M. 1977. Photoperiodism in higher plants: an interaction of phytochrome and circadian rhythms. *Physiol. Plant* 39: 25-32.
- Heimann, K., G. Kreimer, M. Melkonian, and E. Latzko. 1986. Light-induced Ca^{2+} influx into spinach protoplasts. *Plant Physiol* 80: 588.
- Heldt, H.W. 1979. Light dependant changes of stroma H^+ and Mg^{2+} concentrations controlling CO_2 fixation. Pp. 202-207 in *Encyclopedia of plant physiology* (M. Gibbs and E. Latzko, Eds.). Vol. 6. Berlin, Springer-Verlag.
- Heldt, H.W., K. Werdan, M. Milovancev, and G. Geller. 1973. Alkalization of the chloroplast stroma caused by light-dependent proton flux into the thylakoid space. *Biochem. Biophys. Acta* 314: 224-241.
- Hemmings, B.A. 1982. The mechanism, role and control of the inactivation of glutamate dehydrogenases in yeast. *Biochem. Soc. Trans* 10: 328-329.
- Hendrickson, H.R. and E.E. Conn. 1969. Cyanide metabolism in plants. IV. Purification and properties of β -cyanoalanine synthase of blue lupine. *J. Biol. Chem.* 244: 2632-2640.
- Hersh, L.B. 1971. Rat liver α -amidase. Purification and properties. *Biochem.* 10: 2884-2891.
- Hertig, C. and R.A. Wolosiuk. 1980. A dual effect of Ca^{2+} on chloroplast fructose-1,6-bisphosphatase. *Biochem. Biophys. Res. Comm* 97: 325-333.
- Hirel, B., J. Vidal, and P. Gadal. 1982. Evidence for a cytosolic-dependent light induction of chloroplastic glutamine synthetase during greening of etiolated rice leaves. *Planta* 155: 17-23.
- Horowitz, B. and A. Meister. 1972. Glutamine-dependent asparagine synthetase from leukemia cells. *J. Biol. Chem.* 247: 6708-6719.

- Huber, T.A. and J.G. Streeter. 1985. Purification and properties of asparagine synthetase from soybean root nodules. *Plant Sci.* 42: 9-17.
- Hucklesby, D.P., M.J. Dowling, and E.J. Hewitt. 1982. Cyanide formation from glyoxylate and hydroxylamine catalysed by extracts of higher-plant leaves. *Planta* 156: 487-491.
- Ikeda, M., W.L. Ogren, and R.H. Hageman. 1984. Effect of methionine sulfoximine on photosynthetic carbon metabolism in wheat leaves. *Plant Cell Physiol.* 25: 447-452.
- Imada, A., S. Igarasi, K. Nakahama, and M. Isono. 1973. Asparaginase and glutaminase activities of micro-organisms. *J. Gen. Microbiol.* 76: 85-99.
- Ireland, R.J. and K.W. Joy. 1983a. Purification and properties of an asparagine aminotransferase from *Pisum sativum* leaves. *Arch. Biochem. Biophys.* 223: 291-296.
- Ireland, R.J. and K.W. Joy. 1983b. Subcellular localization of asparaginase and asparagine aminotransferase in *Pisum sativum* leaves. *Plant. Physiol.* 72: 1127-1129.
- Ireland, R.J. and K.W. Joy. 1981. Two routes for asparagine metabolism in *Pisum sativum* L. *Planta* 151: 289-292.
- Ito, O., T. Yoneyama, and K. Kumazawa. 1978. Amino acid metabolism in plant leaf IV. The effect of light on ammonium assimilation and glutamine metabolism in the cells isolated from spinach leaves. *Plant Cell Physiol.* 19: 1109-1119.
- Izawa, S. 1980. Acceptors and donors for chloroplast electron transport. *Meth. Enzymol.* 69: 413-434.

- Jacquot, J-P. 1984. Post-translational modifications of proteins in higher plant chloroplasts: enzyme regulation by thiol-disulfide interchange. *Physiol. Vég.* 22: 487-507.
- Jerebzoﬀ, S. and S. Jerebzoﬀ-Quintin. 1983a. Control of enzyme activity rhythms in *Leptosphaeria michotii*. I. Effects of cycloheximide. *Physiol. Vég.* 21: 233-245.
- Jerebzoﬀ, S. and S. Jerebzoﬀ-Quintin. 1983b. Control of enzyme activity rhythms in *Leptosphaeria michotii*. II. Effects of propargylglycine. *Physiol. Vég.* 21: 247-256.
- Jerebzoﬀ-Quintin, S. and S. Jerebzoﬀ. 1985. L-asparaginase activity in *Leptosphaeria michotii*. Isolation and properties of two forms of the enzyme. *Physiol. Plant.* 64: 74-80.
- Johnson, C.M. and A. Ulrich. 1950. Determination of nitrate in plant material. *Anal. Chem.* 22: 1526-1529.
- Johnson, L.B., C.L. Niblett, and R.F. Lee. 1976. Effects of inhibitors of RNA and protein synthesis on aspartate transcarbamylase activity in etiolated plant tissue. *Plant Physiol.* 58: 232-236.
- Joner, P.E., T. Kristiansen, and M. Einarsson. 1973. Purification and properties of L-asparaginase A from *Acinetobacter calcoaceticus*. *Biochem. Biophys. Acta* 327: 146-156.
- Jones, R.A. 1977. Evidence for cycloheximide acting as a glutamine analogue in plant tissue. *Biochem. Biophys. Acta* 474: 154-161.
- Jordan, B.R. and C.V. Givan. 1979. Effects of light and inhibitors on glutamate metabolism in leaf discs of *Vicia faba* L. *Plant Physiol.* 64: 1043-1047.
- Joy, K.W. 1973. Control of glutamate dehydrogenase from *Pisum sativum* roots. *Phytochem.* 12: 1031-1040.

- Joy, K.W. 1985. Inhibition of asparagine synthetase by calcium. *Plant Physiol.* 77: S 181.
- Joy, K.W. and C. Prabha. 1986. The role of transamination in the synthesis of homoserine in peas. *Plant Physiol.* in press.
- Joy, K.W., R.J. Ireland, and P.J. Lea. 1983. Asparagine synthesis in pea leaves, and the occurrence of an asparagine synthetase inhibitor. *Plant Physiol.* 73: 165-168.
- Kafkewitz, D. and A. Bendich. 1983. Enzyme-induced asparagine and glutamine depletion and immune system function. *Amer. J. Clin. Nutr.* 37: 1025-1030.
- Kakefuda, G., S.H. Duke, and S.O. Duke. 1983. Differential light induction of nitrate reductases in greening and photobleached soybean seedlings. *Plant Physiol.* 73: 56-60.
- Kanazawa, T., M. Distefano, and J.A. Bassham. 1983. Ammonia regulation of intermediary metabolism in photosynthesizing and respiring *Chlorella pyrenoidosa*: comparative effects of methylamine. *Plant Cell Physiol.* 24: 979-986.
- Kauss, H., H. Kohle, and W. Jeblick. 1983. Proteolytic activation and stimulation by Ca^{2+} of glucan synthase from soybean cells. *FEBS Letts.* 158: 84-88.
- Kern, R. and M.J. Chrispeels. 1978. Influence of the axis on the enzymes of protein and amide metabolism in the cotyledons of mung bean seedlings. *Plant Physiol.* 62: 815-819.
- Keys, A.J., M. Bird, M.J. Cornelius, P.J. Lea, R.M. Wallsgrove, and B.J. Milfin. 1978. Photorespiratory nitrogen cycle. *Nature* 275: 741-743.

- Kim, K.W. and R.J. Roon. 1983. Asparaginase II of *Saccharomyces cerevisiae*: comparison of enzyme stability *in vivo* and *in vitro*. Biochem. 22: 2704-2707.
- Kingdon, H.S., B.M. Shapiro, and E.R. Stadtman. 1967. Regulation of glutamine synthetase VIII. Proc. Nat. Acad. Sci. USA. 58: 1703-1710.
- Kloppstech, K. 1985. Diurnal and circadian rhythmicity in the expression of light-induced plant nuclear messenger RNAs. Planta 165: 502-506.
- Knight, T.J. and G.S. Weissman. 1982. Rhythms in glutamine synthetase activity, energy charge, and glutamine in sunflower roots. Plant Physiol. 70: 1683-1688.
- Knoll, H.R. 1978. Association of glycolytic enzymes with particulate fractions from nerve endings. Biochem. Biophys. Acta 522: 1-9.
- Koch, F.C. and T.L. McMeekin. 1924. A new direct nesslerization micro-kjeldahl method and a modification of the Nessler-Folin reagent for ammonia. J. Amer. Chem. Soc. 46: 2066-2069.
- Kreimer, G., M. Melkonian, J.A.M. Holtum, and E. Latzko. 1985. Characterization of calcium fluxes across the envelope of intact spinach chloroplasts. Planta 166: 515-523.
- Kretovich, W.L. 1958. The biosynthesis of dicarboxylic amino acids and enzymic transformations of amides in plants. Adv. in Enzymol. 20: 319-340.
- Kumar, P.A., T.V.R. Nair, and Y.P. Abrol. 1984. Effect of photorespiratory metabolites, inhibitors and methionine sulfoximine on the accumulation of ammonia in the leaves of mung bean and *Amaranthus*. Plant Sci. Letts. 33: 303-307.

- Lamb, C.J. and M.A. Lawton. 1983. Photocontrol of gene expression. Pp. 213-257 /n Encyclopedia of plant physiology (W. Shropshire, Jr. and H. Mohr, Eds.). Vol. 16A. Berlin, Springer-Verlag.
- Larsen, P.O. 1980. Physical and chemical properties of amino acids. Pp. 225-269 /n The biochemistry of plants (B.J. Mifflin, Ed.). Vol. 5. New York, Academic Press.
- Larsen, P.O., K.L. Cornwell, S.L. Gee, and J.A. Bassham. 1981. Amino acid synthesis in photosynthesizing spinach cells. Plant Physiol. 68: 292-299.
- Larsson, M., C-M. Larsson, and W.R. Ullrich. 1982. Regulation by amino acids of photorespiratory ammonia and glycolate release from *Ankistrodesmus* in the presence of methionine sulfoximine. Plant. Physiol. 70: 1637-1640.
- Laszlo, J., D. Miller, K. McCarty, and P. Hochstein. 1966. Actinomycin D: inhibition of respiration and glycolysis. Science 151: 1007-1010.
- Latzko, E. and G.J. Kelly. 1979. Enzymes of the reductive pentose phosphate cycle. Pp. 239-250 /n Encyclopedia of plant physiology (M. Gibbs and E. Latzko, Eds.). Vol. 6. Berlin, Springer-Verlag.
- Lea, P.J. and L. Fowden. 1975a. Asparagine metabolism in higher plants. Biochem. Physiol. Pflanzen 168: S. 3-14.
- Lea, P.J. and L. Fowden. 1975b. The purification and properties of glutamine-dependent asparagine synthetase isolated from *Lupinus albus*. Proc. R. Soc. Lond. B. 192: 13-26.
- Lea, P.J. and K.W. Joy. 1983. Amino acid interconversion in germinating seeds. Pp. 77-109 /n Recent advances in phytochemistry (C. Nozzolillo, P.J. Lea and Loewus, Eds.). Vol. 17. New York, Plenum Press.

- Lea P.J. and B.J. Mifflin. 1979. Photosynthetic ammonia assimilation. Pp. 445-456 /n Encyclopedia of plant physiology (M. Gibbs and E. Latzko, Eds.). Vol. 6. Berlin, Springer-Verlag.
- Lea, P.J. and B.J. Mifflin. 1980. Transport and metabolism of asparagine and other nitrogen compounds within the plant. Pp. 569-607 /n The biochemistry of plants (B.J. Mifflin, Ed.). Vol. 5. New York, Academic Press.
- Lea, P.J., L. Fowden, and B.J. Mifflin. 1978. The purification and properties of asparaginase from *Lupinus* species. Biochem. 17: 217-222.
- Lea, P.J., J.S. Hughes, and B.J. Mifflin. 1979. Glutamine- and asparagine-dependent protein synthesis in maturing legume cotyledons cultured *in vitro*. J. Exper. Bot. 30: 529-537.
- Lea, P.J., G.N. Festenstein, J.S. Hughes, and B.J. Mifflin. 1984. An immunological and enzymological survey of asparaginase in seeds of *Lupinus*. Phytochem. 23: 511-514.
- Leegood, R.C. and D.A. Walker. 1982. Regulation of fructose-1,6-bisphosphatase activity in leaves. Planta 156: 449-456.
- Leegood, R.C., G.E. Edwards, and D.A. Walker. 1982. Chloroplasts and protoplasts. Pp. 94-111 /n Techniques in bioproductivity and photosynthesis (J. Coombs and D.O. Hall, Eds.). Oxford, Pergamon Press.
- Lees, E.M. and A.B. Blakeney. 1970. The distribution of asparaginase activity in legumes. Biochim. Biophys. Acta 215: 145-151.
- Lees, E.M., K.J.F. Farnden, and W.H. Elliott. 1968. Studies on asparagine synthesis and utilization in seedlings. Arch. Biochem. Biophys. 126: 539-546.
- Lerman, M.I. and S.P. Mardashev. 1960. Biosynthesis of asparagine from labeled precursors. Biochem. 25: 946-953.

- Lilley, R.McC., M.P. Fitzgerald, K.G. Rienits, and D.A. Walker. 1975. Criteria of intactness and the photosynthetic activity of spinach chloroplast preparations. *New Phytol.* 75: 1-10.
- Lillo, C. 1983. Diurnal variations of nitrate reductase activity and stability in barley leaves. *Physiol. Plant.* 58: 184-188.
- Lillo, C. 1984a. Diurnal variations of nitrite reductase, glutamine synthetase, glutamate synthase, alanine aminotransferase and aspartate aminotransferase in barley leaves. *Physiol. Plant.* 61: 214-218.
- Lillo, C. 1984b. Circadian rhythmicity of nitrate reductase activity in barley leaves. *Physiol. Plant.* 61: 219-223.
- Lillo, C. and A. Henriksen. 1984. Comparative studies of diurnal variations of nitrate reductase activity in wheat, oat and barley. *Physiol. Plant.* 62: 89-94.
- Lloyd, W.D.H. and K.W. Joy. 1978. 2-hydroxysuccinamic acid: a product of asparagine metabolism in plants. *Biochem. Biophys. Res. Comm.* 81: 186-192.
- Macdonald, I.R. and R.J. Ellis. 1969. Does cycloheximide inhibit protein synthesis specifically in plant tissues? *Nature* 222: 791-792.
- Maldonado, J.M., B.A. Notton, and E.J. Hewitt. 1982. The reactivation of nitrate reductase from spinach (*Spinacea oleracea* L.) inactivated by NADH and cyanide: effects of peroxidase and associated systems.
- Marmé, D. 1985. The role of calcium in the cellular regulation of plant metabolism. *Physiol. Vég.* 23: 945-953.

- Marques, I.A., M.J. Oberholzer, and K.H. Erismann. 1983. Effects of different inorganic nitrogen sources on photosynthetic carbon metabolism in primary leaves of nonnodulated *Phaseolus vulgaris* L. Plant Physiol. 71: 555-561.
- Martin, F., M.J. Winspear, J.D. MacFarlane, and A. Oaks. 1983. Effect of methionine sulfoximine on the accumulation of ammonia in C₃ and C₄ leaves. Plant Physiol. 71: 177-181.
- Mashburn, L.T. and J.C. Wriston. 1964. Tumour inhibitory effect of L-asparaginase from *Escherichia coli*. Arch. Biochem. Biophys. 105: 450-452.
- Masters, C. 1985. Phosphofructokinase, compartmentation and the regulation of glycolysis. Trends Biochem. Sci. 10: 189.
- McCullough, H. 1967. The determination of ammonia in whole blood by a direct colorimetric method. Clin. Chim. Acta 17: 297-304.
- McCurdy, D.W. and L.H. Pratt. 1986. Kinetics of intracellular redistribution of phytochrome in *Avena* coleoptiles after its photoconversion to the active, far-red-absorbing form. Planta 167: 330-336.
- McMahon, D. 1975. Cycloheximide is not a specific inhibitor of protein synthesis *in vivo*. Plant Physiol. 55: 815-821.
- McNally, S. and B. Hirel. 1983. Glutamine synthetase isoforms in higher plants. Physiol. Vég. 21: 761-774.
- McNeill, D.L., C.A. Atkins, and J.S. Pate. 1979. Uptake and utilization of xylem borne amino compounds by shoot organs of a legume. Plant. Physiol. 63: 1076-1081.
- Meister, A. 1953. Preparation and enzymatic reactions of the keto analogues of asparagine and glutamine. J. Biol. Chem. 200: 571-590.

- Meister, A. 1954. Studies on the mechanism and specificity of the glutamine- α -keto acid transamination-deamidation reaction. *J. Biol. Chem.* 210: 17-35.
- Meister, A. and P.E. Fraser. 1954. Enzymatic formation of L-asparagine by transamination. *J. Biol. Chem.* 210: 37-43.
- Meister, A. and S.V. Tice. 1950. Transamination from glutamine to α -keto acids. *J. Biol. Chem.* 187: 173-187.
- Meister, A., L. Levintow, R.E. Greenfield, and P. A. Abendschein. 1955. Hydrolysis and transfer reactions catalyzed by α -amidase preparations. *J. Biol. Chem.* 215: 441-460.
- Meister, A., H.A. Sober, S.V. Tice, and P.E. Fraser. 1952. Transamination and associated deamidation of asparagine and glutamine. *J. Biol. Chem.* 197: 319-330.
- Mifflin, B.J. and P.J. Lea. 1975. Glutamine and asparagine as nitrogen donors for reductant dependant glutamate synthesis in pea roots. *Biochem. J.* 149: 403-409.
- Mifflin, B.J. and P.J. Lea. 1980. Ammonia assimilation. Pp. 169-202. *In* The biochemistry of plants (B.J. Mifflin, Eds.). Vol. 5. New York, Academic Press.
- Mifflin, B.J. and P.J. Lea. 1982. Ammonia assimilation and amino acid metabolism. Pp. 5-64 *In* Encyclopedia of plant physiology (D. Boulter and B. Parthier, Eds.). Vol. 14A. Berlin, Springer-Verlag.
- Mills, W.R. and B.J. Baumgartner. 1983. Light-driven DNA biosynthesis in isolated pea chloroplasts. *FEBS Letts.* 163: 124-127.
- Mills, W.R. and K.W. Joy. 1980. A rapid method for isolation of purified, physiologically active chloroplasts, used to study the intracellular distribution of amino acids in pea leaves. *Planta* 148: 75-83.

- Mills, W.R., P.J. Lea, and B.J. Mifflin. 1980. Photosynthetic formation of the aspartate family of amino acids in isolated chloroplasts. *Plant Physiol.* 65: 1166-1172.
- Mizobuchi, M., K. Tamase, Y. Kitada, M. Sasaki, and K. Tanigawa. High-performance liquid chromatographic analysis of ammonium in human urine. *Anal. Biochem.* 137: 88-92.
- Mohamed, A.H. and A. Gnanam. 1979. A possible mechanism of ammonium ion regulation of photosynthetic carbon flow in higher plants. *Plant Physiol.* 64: 263-268.
- Mohr, H. 1984. Criteria for photoreceptor involvement. Pp. 13-42 *in* Techniques in photomorphogenesis (H. Smith and M.G. Holmes, Eds.). New York, Academic Press.
- Moore, A.L. and K.E.O. Åkerman. 1984. Calcium and plant organelles. *Plant, Cell, Environ.* 7: 423-429.
- Mosinger, E. and P. Schopfer. 1983. Polysome assembly and RNA synthesis during phytochrome-mediated photomorphogenesis in mustard cotyledons. *Planta* 158: 501-511.
- Muftic, M.K. 1964. A new phenol-hypochlorite reaction for ammonia. *Nature* 201: 623-624.
- Murray, D.R. and I.R. Kennedy. 1980. Changes in activities of enzymes of nitrogen metabolism in seedcoats and cotyledons during embryo development in pea seeds. *Plant Physiol.* 66: 782-786.
- Naik, M.S. and D.J.D. Nicholas. 1986. Malate metabolism and its relation to nitrate assimilation in plants. *Phytochem.* 25: 571-576.
- Nauen W. and T. Hartmann. 1980. Glutamate dehydrogenase from *Pisum sativum* L. *Planta* 148: 7-16.

- Nazar, B.L. and A. C. Schoolwerth. 1979. An improved microfluorometric enzymatic assay for the determination of ammonia. *Anal. Biochem.* 95: 507-511.
- Nicholas, J.C., J.E. Harper, and R.H. Hageman. 1976. Nitrate reductase activity in soybeans (*Glycine max* [L.] Merr.). *Plant Physiol.* 58: 731-735.
- Nishizawa, A.N., R.A. Wolosiuk, and B.B. Buchanan. 1979. Chloroplast phenylalanine ammonia-lyase from spinach leaves. *Planta* 145: 7-12.
- Noguchi, T. and S. Hayashi. 1980. Peroxisomal localization and properties of tryptophan aminotransferase in plant leaves. *J. Biol. Chem.* 255: 2267-2269.
- Oaks, A. and D.W. Ross. 1984. Asparagine synthetase in *Zea mays*. *Can. J. Bot.* 62: 68-73.
- O'Neal, T.D. and K.W. Joy. 1975. Pea leaf glutamine synthetase. *Plant Physiol.* 55: 968-974.
- Papageorgiou, G. 1975. Chlorophyll fluorescence: an intrinsic probe of photosynthesis. Pp. 319-371 // *Bioenergetics of photosynthesis* (Govindjee, Ed.). New York, Academic Press.
- Patton, C.J. and S.R. Crouch. 1977. Spectrophotometric and kinetics investigation of the Berthelot reaction for the determination of ammonia. *Anal. Chem.* 49: 464-469.
- Pate, J.S. 1973. Uptake, assimilation and transport of nitrogen compounds by plants. *Soil Biol. Biochem.* 5: 109-119.
- Pate, J.S. 1980. Transport and partitioning of nitrogenous solutes. *Ann. Rev. Plant Physiol.* 31: 313-340.

- Pate, J.S. 1983a. Distribution of metabolites. Pp. 335-401 /n Plant physiology (F.C. Steward and R.G.S. Bidwell, Eds.). Vol. VIII. New York, Academic Press.
- Pate, J.S. and C.A. Atkins. 1983b. Nitrogen uptake, transport and utilization. Pp. 245-297 /n Ecology of nitrogen fixation (W.J. Broughton, Ed.). Vol. 3. Oxford, Oxford Univ. Press
- Paul, J.H. 1982. Isolation and characterization of a *Chlamydomonas* L-asparaginase. Biochem. J. 201: 109-115.
- Paul, J.H. and K.E. Cooksey. 1979. Asparagine metabolism and asparaginase activity in a euryhaline *Chlamydomonas* species. Can. J. Microbiol. 25: 1443-1451.
- Paul, J.H. and K.E. Cooksey. 1981a. Regulation of L-asparaginase in a *Chlamydomonas* species in response to ambient concentrations of combined nitrogen. J. Bacteriol. 147: 9-12.
- Paul, J.H. and K.E. Cooksey. 1981b. Regulation of asparaginase, glutamine synthetase, and glutamate dehydrogenase in response to medium nitrogen concentrations in a euryhaline *Chlamydomonas* species. Plant Physiol. 68: 1364-1368.
- Peiser, G., T-T. Wang, N. Hoffman, and S.F. Yang. 1983. Evidence for $^{14}\text{CN}^-$ formation from $[1-^{14}\text{C}]$ ACC during *in vivo* conversion of ACC to ethylene. Plant Physiol. 72: S 37.
- Peterson, R.B. 1982. Regulation of glycine decarboxylase and L-serine hydroxymethyltransferase activities by glyoxylate in tobacco leaf mitochondrial preparations. Plant Physiol. 70: 61-66.
- Pistorius, E.K., H-S. Gewitz, H. Voss, and B. Vennesland. 1976. Reversible inactivation of nitrate reductase in *Chlorella vulgaris* *in vivo*. Planta 128: 73-80.

- Platt, S.G. and G.E. Anthon. 1981. Ammonia accumulation and inhibition of photosynthesis in methionine sulfoximine treated spinach. *Plant Physiol.* 67: 509-513.
- Platt, S.G. and L. Rand. 1982. Methionine sulfoximine effects on C_4 plant leaf discs: comparison with C_3 species. *Plant Cell Physiol* 23: 917-921.
- Platt, S.G., Z. Plaut, and J.A. Bassham. 1977. Ammonium regulation of carbon metabolism in photosynthesizing leaf discs. *Plant Physiol.* 60: 739-742.
- Portis, A.R. Jr. and H.W. Heldt. 1976. Light dependent changes of the Mg^{2+} concentration in the stroma in relation to the Mg^{2+} dependency of CO_2 fixation in intact chloroplasts. *Biochem. Biophys. Acta* 449: 434-446.
- Pradet, A. and P. Raymond. 1983. Adenine nucleotide ratios and adenylate energy charge in energy metabolism. *Ann. Rev. Plant Physiol.* 34: 199-224.
- Prager, M.D., F.S. Baechtel, and A. Helfetz. 1982. Antitumour and other effects of asparaginase. Pp. 291-306 */n* *Bacteria and cancer* (Jeljaszewicz, Pulverer, and Roszkowski, Eds.).
- Prusiner, S. and L. Milner. 1970. A rapid radioactive assay for glutamine synthetase, glutaminase, asparagine synthetase, and asparaginase. *Anal. Biochem.* 37: 429-438.
- Puritch, G.S. and A.V. Barker. 1967. Structure and function of tomato leaf chloroplasts during ammonium toxicity. *Plant Physiol.* 42: 1229-1238.
- Quail, P.H. 1983. Rapid action of phytochrome in photomorphogenesis. Pp. 178-212 */n* *Encyclopedia of plant physiology* (W. Shropshire, Jr. and H. Mohr, Eds.). Vol. 16A. Berlin, Springer-Verlag.

- Rao, K.P. and D.D. Randall. 1980. Plant pyruvate dehydrogenase complex: inactivation and reactivation by phosphorylation and dephosphorylation. Arch. Biochem. Biophys. 200: 461-466
- Ranjeva, R., A. Graziana, B. Ranty, G. Cavalié, and A.M. Boudet. 1984. Phosphorylation of proteins in plants: a step in the integration of extra and intracellular stimuli? Physiol. Vég. 22: 365-376
- Reed, A.J. and D.T. Canvin. 1982. Light and dark controls of nitrate reduction in wheat (*Triticum aestivum* L.) protoplasts. Plant Physiol. 69: 508-513.
- Refeno, G., R. Ranjeva, and A.M. Boudet. 1982. Modulation of quinate: NAD⁺ oxidoreductase activity through reversible phosphorylation in carrot cell suspensions. Planta 154: 193-198.
- Reilly, C., G-F Fuhrmann, and A. Rothstein. 1970. The inhibition of K⁺ and phosphate uptake in yeast by cycloheximide. Biochem. Biophys. Acta 203: 583-585.
- Resnick, A.D. and B. Magasanik. 1976. L-asparaginase of *Klebsiella aerogenes*: activation of its synthesis by glutamine synthetase. J. Biol. Chem. 251: 2722-2728.
- Robinson, S.P. and W.J.S. Downton. 1984. Potassium, sodium, and chloride content of isolated intact chloroplasts in relation to anionic compartmentation in leaves. Arch. Biochem. Biophys. 228: 197-206
- Rognes, S.E. 1975. Glutamine-dependent asparagine synthetase from *Lupinus luteus*. Biochem. 14: 1975-1982.
- Rognes, S.E. 1980. Anion regulation of lupin asparagine synthetase: chloride activation of the glutamine-utilizing reactions. Phytochem. 19: 2287-2293.

4 OF 4

MICROCOPY RESOLUTION TEST CHART
NATIONAL BUREAU OF STANDARDS
STANDARD REFERENCE MATERIAL 1010a
(ANSI and ISO TEST CHART No. 2)

- Roen, R.J., M. Murdoch, B. Kunze, and P.C. Dunlop. 1982. Derepression of asparaginase II during exponential growth of *Saccharomyces cerevisiae* on ammonium ion. Arch. Biochem. Biophys. 219: 101-109.
- Rosa, L. and F.R. Whatley. 1981. Uncouplers stimulate photosynthesis in intact chloroplasts by enhancing light-activation of enzymes regulated by the ferredoxin-thioredoxin system. Plant Physiol. 68: 364-370.
- Roux, S.J., R.O. Wayne, and N. Datta. 1986. Role of calcium ions in phytochrome responses: an update. Physiol Plant. 66: 344-348.
- Russel, J.A. 1944. The colorimetric estimation of small amounts of ammonia by the phenol-hypochlorite reaction. J. Biol. Chem. 156: 457-461.
- Sane, P.V., S. Kochhar, N. Kumar, and V.K. Kochhar. 1984. Activation of plant aspartate kinase by calcium and calmodulin-like factor from plants. FEBS Letts. 175: 238-242.
- Sarojini, G. and D.J. Oliver. 1985. Inhibition of glycine oxidation by carboxymethoxylamine, methoxylamine, and acethydrazide. Plant Physiol. 77: 786-789.
- Sawheny, S.K. and M.S. Naik. 1973. Effect of chloramphenicol and cycloheximide on the synthesis of nitrate reductase and nitrite reductase in rice leaves. Biochem. Biophys. Res. Comm. 51: 67-73.
- Sawhney, S.K. and M.S. Naik. 1972. Role of light in the synthesis of nitrate reductase and nitrite reductase in rice seedlings. Biochem. J. 130: 475-485.
- Sawhney, S.K., M.S. Naik, and D.J.D. Nicholas. 1978. Regulation of nitrate reduction by light, ATP and mitochondrial respiration in wheat leaves. Nature 272-273.

- Scheibe, R. and L.E. Anderson. 1981. Dark modulation of NADP-dependent malate dehydrogenase and glucose-6-phosphate dehydrogenase in the chloroplast. *Biochem. Biophys. Acta* 636: 58-64.
- Scheibe, R. and E. Beck. 1979. On the mechanism of activation by light of the NADP-dependent malate dehydrogenase in spinach chloroplasts. *Plant Physiol.* 64: 744-748.
- Schrader, L.E., L. Beevers, and R.H. Hageman. 1967. Differential effects of chloramphenicol on the induction of nitrate and nitrite reductase in green leaf tissue. *Biochem. Biophys. Res. Comm.* 26: 14-17.
- Schwall, M., H. Wendorff, and E. Wagner. 1985. Diurnal modulation of arginine decarboxylase activity of *Chenopodium rubrum* L. by temperature and light. *Plant Cell Physiol.* 26: 1607-1612.
- Scott, D.B. and K.J.F. Farnden. 1976. Ammonia assimilation in lupin nodules. *Nature* 263: 703-705.
- Sharkey, P.J. and J.S. Pate. 1975. Selectivity in xylem to phloem transfer of amino acids in fruiting shoots of white lupin (*Lupinus albus* L.). *Planta* 127: 251-262.
- Shelp, B.J., K. Siecichowicz, R.J. Ireland, and K.W. Joy. 1985. Determination of urea and ammonia in leaf extracts: application to ureide metabolism. *Can. J. Bot.* 63: 1135-1140.
- Shen, T.C. 1972. Variation in the nitrate reductase of rice seedlings. *Planta* 108: 21-28.
- Sherrard, J.H., J.A. Kennedy, and M.J. Dalling. 1979. *In vitro* stability of nitrate reductase from wheat leaves. *Plant Physiol.* 64: 439-444.
- Siecichowicz, K.A., R.J. Ireland, and K.W. Joy. 1985. Diurnal variation of asparaginase in developing pea leaves. *Plant Physiol.* 77: 506-508.

- Sihag, R.K., S. Guha-Mukherjee, and S.K. Sopory. 1979. Effect of ammonium, sucrose and light on the regulation of nitrate reductase level in *Pisum sativum*. *Physiol. Plant.* 45: 281-287.
- Singh, P., P.A. Kumar, Y.P. Abrol, and M.S. Naik. 1985. Photorespiratory nitrogen cycle - a critical evaluation. *Physiol. Plant.* 66: 169-176.
- Skokut, T.A., J.E. Varner, J. Schaefer, E.O. Stejskal, and R.A. McKay. 1982. [^{15}N]NMR determination of asparagine and glutamine nitrogen utilization for synthesis of storage protein in developing cotyledons of soybean in culture. *Plant Physiol.* 69: 308-313.
- Smarrelli, J., Jr. and W.H. Campbell. 1983. Heavy metal inactivation and chelator stimulation of higher plant nitrate reductase. *Biochem. Biophys. Acta.* 742: 435-445.
- Smith, H. 1976. Phytochrome-mediated assembly of polyribosomes in etiolated bean leaves. *Eur. M. Biochem.* 65: 161-170.
- Smith, H., E.E. Billett, and A.B. Giles. 1977. The photocontrol of gene expression in higher plants. Pp. 93-127 *in* Regulation of enzyme synthesis and activity in higher plants (H. Smith, Ed.). New York, Academic Press.
- Sodek, L., P.J. Lea, and B.J. Mifflin. 1980. Distribution and properties of a potassium-dependent asparaginase isolated from developing seeds of *Pisum sativum* and other plants. *Plant Physiol.* 65: 22-26.
- Solomonson, L.P. and A.M. Spehar. 1977. Model for the regulation of nitrate assimilation. *Nature* 265: 373-375.
- Somers, D.A., T-M. Kua, A. Kleinhofs, R.L. Warner, and A. Oaks. 1983. Synthesis and degradation of barley nitrate reductase. *Plant Physiol.* 72: 949-952.

- Srivastava, H.S. 1980. Regulation of nitrate reductase activity in higher plants. *Phytochem.* 19: 725-733.
- Steward, F.C. and D.J. Durzan. 1965. Metabolism of organic nitrogenous compounds. Pp. 379-686 /in/ *Plant physiology* (F.C. Steward, Ed.). Vol. 4A. New York, Academic Press.
- Stewart, G.R., A.F. Mann, and P.A. Fentem. 1980. Enzymes of glutamate formation: glutamate dehydrogenase, glutamine synthetase, and glutamate synthase. Pp. 271-328 /in/ *Biochemistry of plants* (B.J. Mifflin, Eds.). Vol. 5. New York, Academic Press.
- Stitt, M., W. Wirtz, R. Gerhardt, H.W. Heldt, Ca. Spencer, D. Walker, and Ch. Foyer. 1985. A comparative study of metabolite levels in plant leaf material in the dark. *Planta* 166: 354-364.
- Streeter, J.G. 1973. *In vivo* and *in vitro* studies on asparagine biosynthesis in soybean seedlings. *Arch. Biochem. Biophys.* 157: 613-624.
- Streeter, J.G. 1977. Asparaginase and asparagine transaminase in soybean leaves and root nodules. *Plant Physiol.* 60: 235-239.
- Streit, L. and U. Feller. 1983. Nitrogen-metabolizing enzymes from bean leaves (*Phaseolus vulgaris* L.): stability *in vitro* and susceptibility to proteolysis. *Z. Pflanzenphysiol. Bd.* 111: 19-27.
- Stulen, I., G.F. Israelstam, and A. Oaks. 1979. Enzymes of asparagine synthesis in maize roots. *Planta* 146: 237-241.
- Surek, B., G. Kreimer, L. Lukow, J.A.M. Holtum, M. Melkonian, and E. Latzko. 1986. Calcium uptake into spinach chloroplasts. *Plant Physiol.* 80: S 88.

Suzuki, A. and P. Gadal. 1984. Glutamate synthase: physicochemical and functional properties of different forms in higher plants and in other organisms. *Physiol. Vég.* 22: 471-486.

Ta, T.C. and K.W. Joy. 1985. Transamination, deamidation, and the utilisation of asparagine amino nitrogen in pea leaves. *Can. J. Bot.* 63: 881-884.

Ta, T.C. and K.W. Joy. 1986. Metabolism of some amino acids in relation to the photorespiratory nitrogen cycle of pea leaves. *Planta* in press.

Ta, T.C., K.W. Joy, and R.J. Ireland. 1984a. Amino acid metabolism in pea leaves. *Plant Physiol.* 74: 822-826.

Ta, T.C., K.W. Joy, and R.J. Ireland. 1984b. Utilization of the amide groups of asparagine and 2-hydroxysuccinamic acid by young pea leaves. *Plant Physiol.* 75: 527-530.

Ta, T.C., K.W. Joy, and R.J. Ireland. 1985. Role of asparagine in the photorespiratory nitrogen metabolism of pea leaves. *Plant Physiol.* 78: 334-337.

Tate, S.S. and A. Meister. 1973. Glutamine synthetases of mammalian liver and brain. Pp. 77-127 *in* The enzymes of glutamine metabolism (S. Prusiner and E.R. Stadtman, Eds.). New York, Academic Press.

Tate, S.S. and A. Meister. 1975. Identity of maleate-stimulated glutaminase with γ -glutamyl transpeptidase in rat kidney. *J. Biol. Chem.* 250: 4619-4627.

Tetlow, J.A. and A.L. Wilson. 1964. An absorptiometric method for determining ammonia in boiler feed-water. *Analyst.* 89: 453-465.

Ting, I.P. and W.C. Zschoche. 1970. Asparagine biosynthesis by cotton roots - carbon dioxide fixation and cyanide incorporation. *Plant Physiol.* 45: 429-434.

- Tischner, R. and A. Schmidt. 1982. A thioredoxin-mediated activation of glutamine synthetase and glutamate synthase in synchronous *Chlorella sorokiniana*. Plant Physiol. 70: 113-116.
- Tobin, E.M. and J. Silverthorne. 1985. Light regulation of gene expression in higher plants. Ann. Rev. Plant Physiol. 36: 569-593.
- Tobin, A.K., S.M. Ridley, and G.R. Stewart. 1985. Changes in the activities of chloroplast and cytosolic isoenzymes of glutamine synthetase during normal leaf growth and plastid development in wheat. Planta 163: 544-548.
- Tolbert, N.E. 1980. Photorespiration. Pp. 488-523 // The biochemistry of plants (D.D. Davies, Ed.). Vol. 2. New York, Academic Press.
- Travis, R.L. and J.L. Key. 1971. Correlation between polyribosome level and the ability to induce nitrate reductase in dark-grown corn seedlings. Plant Physiol. 48: 617-620.
- Trebst, A. 1980. Inhibitors of electron flow: tools for the functional and structural localization of carriers and energy conservation sites. Meth Enzymol. 69: 675-715.
- Trewavas, A. 1976. Post-translational modification of proteins by phosphorylation. Ann. Rev. Plant Physiol. 27: 349-374.
- Turner, J.F. and D.H. Turner. 1980. The regulation of glycolysis and the pentose phosphate pathway. Pp. 279-316 // The biochemistry of plants (D.D. Davies, Ed.). Vol 2. New York, Academic Press.
- Urquhart, A.A. and K.W. Joy. 1982. Transport, metabolism, and redistribution of xylem-borne amino acids in developing pea shoots. Plant Physiol. 69: 1226-1232.
- Vanselow, A.P. 1940. Preparation of Nessler's reagent. Ind. Eng. Chem. Anal. Ed. 12: 516-517.

- Veigl, M.L., T.C. Vanaman, and W.D. Sedwick. 1984. Calcium and calmodulin in cell growth and transformation. *Biochem. Biophys. Acta* 738: 21-48.
- Veluthambi, K. and B.W. Poovaiah. 1984. Calcium-promoted protein phosphorylation in plants. *Science* 223: 167-169.
- Wade, H.E., H.K. Robinson, and B.W. Phillips. 1971. Asparaginase and glutaminase activities of bacteria. *J. Gen. Microbiol.* 69: 299-312.
- Wagner, E. 1977. Molecular basis of physiological rhythms. Pp. 33-72 *in* Integration of activity in the higher plant (D.H. Jennings, Ed.). Cambridge, Cambridge Univ. Press.
- Walker, K.A., C.V. Givan, and A.J. Keys. 1984a. Glutamic acid metabolism and the photorespiratory nitrogen cycle in wheat leaves. *Plant Physiol.* 75: 60-66.
- Walker, K.A., A.J. Keys, and C.V. Givan. 1984b. Effect of L-methionine sulfoximine on the products of photosynthesis in wheat (*Triticum aestivum*) leaves. *J. Exp. Bot.* 35: 1800-1810.
- Wallace, W. 1977. Proteolytic inactivation of enzymes. Pp. 177-195 *in* Regulation of enzyme synthesis and activity in higher plants (H. Smith, Ed.). New York, Academic Press.
- Wallsgrave, R.M., P.J. Lea, and B.J. Mifflin. 1982. The development of NAD(P)H-dependent and ferredoxin-dependent glutamate synthase in greening barley and pea leaves. *Planta* 154: 473-476.
- Wallsgrave, R.M., A.J. Keys, P.J. Lea, and B.J. Mifflin. 1983. Photosynthesis, photorespiration and nitrogen metabolism. *Plant, Cell, Environ.* 6: 301-309.
- Walton, N.J. and H.W. Woolhouse. 1986. Enzymes of serine and glycine metabolism in leaves and non-photosynthetic tissues of *Pisum sativum* L. *Planta* 167: 119-128.

- Webster, G.C. and J.E. Varner. 1955. Aspartate metabolism and asparagine synthesis in plant systems. *J. Biol. Chem.* 215: 91-99.
- Werdan, K., H.W. Heldt, and M. Milovancev. 1975. The role of pH in the regulation of carbon fixation in the chloroplast stroma. Studies on CO_2 fixation in the light and dark. *Biochem. Biophys. Acta* 396: 276-292.
- Wilson, D.G., K.W. King, and R.H. Burris. 1954. Transamination reactions in plants. *J. Biol. Chem.* 208: 863-874.
- Wintermans, J.F.G.M. and A. De Mots. 1965. Spectrophotometric characteristics of chlorophylls *a* and *b* and their pheophytins in ethanol. *Biochem. Biophys. Acta* 109: 448-453.
- Wolosiuk, R.A. and B.B. Buchanan. 1977. Thioredoxin and glutathione regulate photosynthesis in chloroplasts. *Nature* 266: 565-567.
- Wolosiuk, R.A., C.M. Hertig, A.N. Nishizawa, and B.B. Buchanan. 1982. Enzyme regulation in C_4 photosynthesis. *FEBS Lett.* 140: 31-35.
- Woo, K.C. and D.T. Canvin. 1980. Effect of ammonia on photosynthetic carbon fixation in isolated spinach leaf cells. *Can. J. Bot.* 58: 505-510.
- Wriston, J.C., Jr. 1985. Asparaginase. Pp. 608-618 *in* Methods in enzymology (A. Meister, Ed.). Vol. 113. New York, Academic Press.
- Wriston, J.C. and T.O. Yellin. 1973. L-asparaginase: a review. *Adv. Enzymol.* 39: 185-248.
- Wurtele, E.S., B.J. Nikolau, and E.E. Conn. 1984. Tissue distribution of β -cyanoalanine synthase in leaves. *Plant Physiol.* 75: 979-982.
- Wyn Jones, R.G. and A. Pollard. 1983. Proteins, enzymes and inorganic ions. Pp. 528-562 *in* Encyclopedia of plant physiology (A. Lauchli and R.L. Bielecki, Eds.). Vol. 15B. Berlin, Springer-Verlag.

Yamamoto, Y. 1955. Asparagine metabolism in the germination stage of a bean, *Vigna sesquipedalis*. J. Biochem. 42: 763-774.

Yamaya, T. and H. Matsumoto. 1985. Ammonia content in mitochondria isolated from detached pea shoots. Effect of light and inhibitors of photorespiratory nitrogen cycle. Plant Cell Physiol. 26: 1613-1616.

Appendix I Hoaglands Nutrient solution (one half normal strength):

<u>element</u>	<u>mM</u>
N as NO_3^-	4.0
NH_4^+	0.009
K	2.0
Ca	1.5
P	1.0
S	1.0
Mg	1.0
	<u>μM</u>
Fe	9.0
B	20.0
Mn	3.8
Zn	0.4
Mo	0.002
Cl	4.2

<u>Compound</u>	<u>stock conc.</u>	<u>add: g/L</u>	<u>use: ml/L</u>
$\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$	1M	236.2	1.5
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	1M	246.5	1.0
KNO_3	1M	101.1	1.0
KH_2PO_4	1M	136.1	1.0
$\text{Fe}(\text{NH}_4)\text{SO}_4 \cdot 6\text{H}_2\text{O}$		1.0	3.6
Versenol		2.1	
H_3BO_4		2.5	
$\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$		1.5	0.5
ZnCl		0.1	
$(\text{NH}_4)_6\text{MO}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$		0.05	

Appendix 2

Reversible inhibition of photosynthesis by DCMU

A range of concentrations of DCMU or atrazine were fed to half expanded leaves for 2.5 hours in the light. Extracts were obtained at the end of the dark period (0 hour) and after 2.5 hours of feeding in the light and assayed for asparaginase activity (figures 21, 22) as well as photosynthetic activity (table appendix.1). An increase in asparaginase activity was observed in the control treatments while no increase in activity was observed in any of the inhibitor treatments above 10 μ M concentration. Photosynthetic activity assayed by the method of DCPIP reduction did not decrease in chloroplast preparations obtained from extracts fed increasing DCMU concentrations, and only a slight decrease in photosynthetic activity was noted in the atrazine treatments (table appendix.1). However, photosynthetic activity was observed to decrease when measured by *in vivo* fluorescence, or O_2 evolution (e.g. figure 20)

The variability observed in the inhibition of photosynthesis measured *in vitro* was thought to be due to removal or dilution of DCMU from the photosystem II complex during chloroplast extraction. To examine this possibility DCMU was added to crude chloroplast preparations which were then washed in buffer several times. After each wash photosynthetic activity was assayed by DCPIP reduction or by O_2 evolution. Earlier experiments, examining the concentrations of herbicide needed to inhibit

Table appendix.1 Comparison of *in vitro* and *in vivo* effects of DCMU and atrazine on photosynthetic activity. Detached half expanded leaves were supplied a range of DCMU or atrazine concentrations for 2.5 hours in the light. Photosynthetic activity was estimated by *in vivo* chlorophyll *a* fluorescence, or *in vitro*, by assaying DCPIP reduction in crude chloroplast preparations. Values are presented as a percent of the control treatment (0 μ M DCMU or atrazine). For asparaginase activity see figure 18.

μ M	atrazine		μ M	DCMU	
	<i>in vitro</i> % control	<i>in vivo</i> treatment		<i>in vitro</i> % control	<i>in vivo</i> treatment
0	100	100	0	100	100
10	100	-	10	116	-
20	73	81	20	93	24
30	76	-	30	107	-
50	79	49	50	101	29

in vitro photosynthesis indicated that a 90% reduction of photosynthesis would be expected in the presence of 1 μM DCMU in assay (see figure 18)

DCMU was added to crude chloroplast preparations to a final concentration of 1 μM and left for 10 minutes at 4°C. A sample of this preparation was then added to the DCPIP assay solution and a 60 to 62% inhibition of photosynthetic activity was observed compared to the untreated control. Photosynthetic activity was inhibited by 58% following a longer incubation at 22°C in 1 μM DCMU for 30 minutes (table appendix.2A). For both of these treatments the final concentration of DCMU present during assay was calculated to be 0.08 μM (table appendix.2A) and this concentration would have produced about a 50 to 60% inhibition of photosynthesis (figure 18).

When chloroplast preparations treated with 1 μM DCMU for 10 minutes were washed by sedimentation and resuspension, the photosynthetic activity increased, and only 15 to 25% inhibition of DCPIP reduction was observed; a second washing did not increase photosynthetic activity (table appendix.2A). When these washed chloroplast preparations were again assayed with DCMU added to give a final concentration of 1 μM in the assay, over 90% inhibition of DCPIP reduction was observed.

Chloroplast preparations were incubated in 1, 2, and 10 μM DCMU and assayed for photosynthetic activity by O_2 evolution. With higher levels of DCMU in the incubation mixture an increase in the inhibition of photosynthesis was noted (up to 91%, table appendix.2B). However, when chloroplast preparations at 10 μM DCMU were washed a partial recovery in photosynthetic activity was observed. If DCMU was added to the washed

Table appendix.2 Effect of washing on DCMU inhibition of photosynthesis in chloroplasts. Crude chloroplast preparations were assayed for photosynthetic activity (control activity) and DCMU added to a final concentration of 1 μM (unless otherwise stated). The mixtures were left for 10 minutes at 4°C (unless otherwise stated) and then centrifuged and resuspended in extraction buffer; this procedure was repeated a second time. Samples were removed from the initial treatment and after each wash assayed for photosynthetic activity by DCPIP reduction (A) or O_2 evolution (B).

(A) treatment	[DCMU] μM in assay	% inhibition DCPIP reduction	
control ^a	0	0	0
1 μM DCMU final.			
incubate 10 min 4°C ^a	0.08	62	60
incubate 30 min 22°C	0.08	58	-
1 st wash ^a	-	24	15
2 nd wash ^a	-	24	13

(B) treatment	[DCMU] μM in assay	% inhibition O_2 evolution	
control ^a	0	0	
1 μM DCMU ^a	0.06	26	
2 μM DCMU ^a	0.12	58	
10 μM DCMU ^a	0.62	91	
1 st wash ^a	-	80	
2 nd wash ^a	-	75	

a) When these preparations were assayed with the addition of DCMU to give a final concentration of 1 μM , photosynthetic activity was inhibited 92 to 94% (for both DCPIP reduction and O_2 evolution assays).

preparations so that the final assay concentration was $1\mu\text{M}$, 92-94% inhibition of photosynthetic activity was noted.

These observations suggest that at low concentrations, DCMU binding may be reversible and this may account for the varied photosynthetic rates observed during the feeding experiments

END

14.08.87

FIN