

THE SHORT TERM EFFECTS OF THE SUPPLY OF INORGANIC
NITROGEN ON THE PHOTOSYNTHETIC ASSIMILATION OF CARBON
DIOXIDE

Michael Denis Cramer

Johannesburg, 1987

THE SHORT TERM EFFECTS OF THE SUPPLY OF INORGANIC
NITROGEN ON THE PHOTOSYNTHETIC ASSIMILATION OF CARBON
DIOXIDE

Michael Denis Cramer

A thesis submitted to the Faculty of Science, University of the
Witwatersrand, in fulfillment of the requirements for the degree
of Master of Science

Johannesburg, 1987

1. ABSTRACT

The short term response (occurring within 0-10 minutes) of photosynthetic carbon dioxide fixation and oxygen evolution to the assimilation of inorganic nitrogen supplied to leaf cells, either as intact tissue, leaf discs or protoplasts, was investigated. The long and short term responses of Lycopersicon esculentum and that of Pisum sativum differed. L. esculentum had higher photosynthetic rates and was considerably more sensitive to N supply. L. esculentum photosynthetic CO_2 assimilation was stimulated by NO_3^- supplied to cut shoots as NaNO_3 in the range 0.1 - 1 mM by up to 20%. Ammonium supplied as NH_4SO_4 (0.5 mM - 1 mM) enhanced carbon assimilation by up to 10%. In P. sativum low concentrations of NO_3^- in the range 0.1 - 10 mM were found to cause an immediate small (5%) increase in photosynthetic carbon fixation. Ammonium concentrations in excess of 0.5 mM were found to inhibit CO_2 fixation in this plant while lower concentrations were stimulatory.

P. sativum protoplasts supplied with nitrate (0.1 - 1mM) showed increased CO_2 fixation while higher concentrations (10 mM) had a depressive effect. Carbon assimilation was inhibited by exogenous nitrite and ammonium. Nitrate, nitrite and ammonium all exhibited the ability to stimulate photosynthetic O_2 evolution from protoplasts at low concentrations. Higher concentrations, ammonium especially, had a limiting effect on O_2 evolution.

The hypotheses advanced to explain these results propose that reductant for NO_3^- assimilation is exported from the chloroplast in the form of DHAP and malate. The NO_3^- stimulated withdrawal of reducing equivalents in the form of DHAP and malate may lead to competition with the Calvin cycle for reductant and intermediates. The chloroplastic reduction of NO_2^- and NH_4^+ may compete with the Calvin cycle for reducing equivalents. Nitrite

acidification of the stroma and NH_4^+ uncoupling and inhibition of photophosphorylation are likely to inhibit CO_2 assimilation. The mechanisms of the observed responses are discussed in the light of these and other hypotheses.

2. DECLARATION

I declare that this thesis is my own, unaided work. It is being submitted for the Degree of Master of Science at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.



Michael Denis Cramer

this 14th day of April 1987.

3. ACKNOWLEDGMENTS

I wish to express my gratitude to my supervisor, Prof. C.F. Cresswell for his supervision and guidance throughout this project.

I am deeply indebted to Dr. V.M. Gray for the many discussions and for his theoretical and practical support.

I wish to acknowledge the financial support of the Council for Scientific and Industrial Research.

4. LIST OF ABBREVIATIONS

ABA	Abscisic acid
ADH	Alcohol dehydrogenase
ADP	Adenosine 5'-diphosphate
AMP	Adenosine 5'-monophosphate
ATP	Adenosine 5'-triphosphate
ATPase	Adenosine 5'-triphosphatase
BSA	Bovine serum albumin
CCCP	Carbonyl cyanide m-chlorophenylhydrazone
CCP	Carbon dioxide compensation point
chl	Chlorophyll
cpm	counts per minute
Cyt	Cytochrome
DCMU	3-(3'-4'-dichlorophenyl)-1,1-dimethyl urea
DHAP	Dihydroxyacetone phosphate
dm	Decimetre
dpm	disintegration per minute
EDTA	Ethylene-dinitrilo tetraacetic acid
equiv.	Reducing equivalents
F6P	Fructose 6-phosphate
FAD	Flavin adenine dinucleotide
Fd(ox)	Ferredoxin oxidised
Fd(rd)	Ferredoxin reduced
Fd	Ferredoxin (unspecified redox state)
Fig.	Figure
FMN(H ₂)	Flavin mono-nucleotide (reduced)
g	gramme
ΔG_0	Change in Gibbs free energy
G6P	Glucose 6-phosphate
GAPDH	Glyceraldehyde phosphate dehydrogenase
GAP	Glyceraldehyde phosphate
Glu	Glutamate
GOGAT	Glutamine: α -ketoglutarate amino transferase
GS	Glutamine synthetase
HEPES	N-2-hydroxyethylpiperazine-N'-ethane sulphonic acid
h	Hours
HMP	Hexose monophosphate shunt
h ν	Light energy
I.R.G.A.	Infra red gas analyzer
K _m	Michaelis-Menton constant
kD	Kilo Daltons
l	Litre
LDH	Lactate dehydrogenase
m	metre
MDH	Malate dehydrogenase
MES	2-(N-morpholino)-ethanesulphonic acid
MFA	Monofluoroacetic acid
mg	Milligramme
min.	Minutes
ml	Millilitre

mM	Millimolar
MSO	Methionine sulfoximine
NAD(+))	Nicotinamide adenine dinucleotide phosphate bispecific
NADH	Nicotinamide adenine dinucleotide reduced
NAD	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate reduced
NADP	Nicotinamide adenine dinucleotide phosphate
NiR	Nitrite reductase
nm	Nanometre
NR	Nitrate reductase
OAA	Oxaloacetic acid
PEPc	Phosphoenol pyruvate carboxylase
PEP	Phosphoenol pyruvate
PFK	Phospho-fructose kinase
PFP	Phospho-fructose phosphatase
PGA	Phosphoglyceric acid
PI	Inorganic phosphate
ppm	parts per million
PQ	Plastoquinone
PS I(I)	Photosystem I(I)
QA	Primary quinone electron acceptor of PS II
QB	Secondary quinone electron acceptor of PS II
Resist.	Resistance
RH	Relative humidity
RuBPC	Ribulose biphosphate carboxylase
RuBPo	Ribulose biphosphate oxygenase
RuBP	Ribulose biphosphate
SAS	Statistical analysis system
S.D.	Standard deviation
S.E.	Standard error
SPS	Sucrose phosphate synthetase
s	Seconds
TCA	Tri-Carboxylic acid cycle
TP	Triose-Phosphate
Triose-P	Triose-Phosphate
[X]	Concentration of substance X
UDP	Uridine 5' -diphosphate
μbar	Micro-bar
μE	Micro-Einsteins
μg	Microgramme
μl	Microlitre
μM	Micro-molar
μmol(es)	Micro-moles
W	Watt
°C	Degree Centigrade

5. TABLE OF CONTENTS

1. ABSTRACT.....	i
2. DECLARATION.....	iii
3. ACKNOWLEDGMENTS.....	iv
4. LIST OF ABBREVIATIONS.....	v
5. TABLE OF CONTENTS.....	vii
6. INTRODUCTION.....	1
7. LITERATURE REVIEW.....	3
7.1. TRANSPORT AND TRANSLOCATION OF NITROGEN.....	3
7.1.1. PLANT ROOT UPTAKE OF INORGANIC NITROGEN...	4
7.1.2. THE TRANSPORT OF NITROGEN ACROSS THE PLASMALEMMMA.....	9
7.1.2.1. Nitrate transport.....	9
7.1.2.2. Ammonium transport.....	15
7.1.2.3. Amino acid transport.....	16
7.1.3. Transport of nitrogen into the vacuole...	17
7.2. NITROGEN EFFECTS ON CARBON METABOLISM THROUGH UPTAKE MECHANISMS.....	21
7.3. ACCUMULATION OF IONS ASSOCIATED WITH INORGANIC NITROGEN REDUCTION.....	24
7.4. EFFECTS OF INORGANIC NITROGEN ON METABOLISM OF OTHER IONS.....	28
7.5. THE EFFECTS OF NITROGEN ON ENZYME ACTIVITIES ASSOCIATED WITH CARBON DIOXIDE ASSIMILATION.....	29
7.6. DIRECT EFFECTS OF INORGANIC NITROGEN ON THE LIGHT REACTION OF PHOTOSYNTHESIS.....	31

THE INTERDEPENDENCE OF INORGANIC NITROGEN REDUCTION AND CARBON METABOLISM.....	32
7.7. REDUCTION OF NITRATE.....	32
7.7.1. Light energy dependent nitrate reduction.....	32
7.7.2. Reduction requirements of Nitrate Reductase.....	33
7.7.2.1. Regulation of Nitrate Reductase.....	36
7.7.2.2. Alternative systems of nitrate reduction.....	37
7.7.3. The source of reductant for Nitrate Reductase.....	38
7.7.3.1. The C ₄ dicarboxylic acid (malate/oxaloacetate) shuttle.....	41
7.7.3.2. The PGA/DHAP shuttle.....	43
7.7.3.3. Carbon assimilation coupled nitrate reduction.....	46
7.7.3.4. The interdependence of photosynthetic and respiratory carbon metabolism and nitrate assimilation.....	50
7.7.3.5. Role of respiration in nitrate reduction.....	53
7.8. THE REDUCTION OF NITRITE.....	58
7.8.0.1. Reduction requirements of Nitrite Reductase.....	59
7.8.0.2. The source of reductant for Nitrite Reductase.....	60
7.9. THE REDUCTION OF AMMONIUM.....	63
7.9.1. The source of reductant for ammonium assimilation.....	65
7.10. THE ROLE OF INORGANIC NITROGEN IONS IN PHOTORESPIRATION.....	68
8. METHODS AND MATERIALS.....	71
8.1. PLANT MATERIAL.....	71
8.1.1. Culture of <i>Lycopersicon esculentum</i>	71
8.1.2. Culture of <i>Pisum sativum</i>	71
8.2. CO ₂ GAS EXCHANGE ANALYSIS.....	73
8.2.1. Photosynthetic CO ₂ fixation.....	73

THE INTERDEPENDENCE OF INORGANIC NITROGEN REDUCTION AND CARBON METABOLISM.....	32
7.7. REDUCTION OF NITRATE.....	32
7.7.1. Light energy dependent nitrate reduction.....	32
7.7.2. Reduction requirements of Nitrate Reductase.....	33
7.7.2.1. Regulation of Nitrate Reductase.....	36
7.7.2.2. Alternative systems of nitrate reduction.....	37
7.7.3. The source of reductant for Nitrate Reductase.....	38
7.7.3.1. The C ₄ dicarboxylic acid (malate/oxaloacetate) shuttle.....	41
7.7.3.2. The PGA/DHAP shuttle.....	43
7.7.3.3. Carbon assimilation coupled nitrate reduction.....	46
7.7.3.4. The interdependence of photosynthetic and respiratory carbon metabolism and nitrate assimilation.....	50
7.7.3.5. Role of respiration in nitrate reduction.....	53
7.8. THE REDUCTION OF NITRITE.....	58
7.8.0.1. Reduction requirements of Nitrite Reductase.....	59
7.8.0.2. The source of reductant for Nitrite Reductase.....	60
7.9. THE REDUCTION OF AMMONIUM.....	63
7.9.1. The source of reductant for ammonium assimilation.....	65
7.10. THE ROLE OF INORGANIC NITROGEN IONS IN PHOTOSYNTHESIS.....	68
8. METHODS AND MATERIALS.....	71
8.1. PLANT MATERIAL.....	71
8.1.1. Culture of <u>Lycopersicon esculentum</u>	71
8.1.2. Culture of <u>Pisum sativum</u>	71
8.2. CO ₂ GAS EXCHANGE ANALYSIS.....	73
8.2.1. Photosynthetic CO ₂ fixation.....	73

8.2.2. Carbon dioxide compensation points.....	77
8.3. STOMATAL RESISTANCE.....	79
8.4. LEAF DISK CARBON DIOXIDE FIXATION.....	79
8.5. PROTOPLAST ISOLATION.....	80
8.6. PROTOPLAST NITRATE, NITRITE AND AMMONIUM UPTAKE STUDIES.....	81
8.6.1. Nitrate.....	82
8.6.1.1. Nitrate net uptake.....	82
8.6.1.2. Dark anaerobic assay for NR.....	83
8.6.1.3. Benzyl viologen assay for NR.....	83
8.6.2. Nitrite net uptake.....	84
8.6.3. Ammonium net uptake.....	84
8.7. PROTOPLAST CARBON DIOXIDE FIXATION.....	85
8.8. PROTOPLAST OXYGEN EVOLUTION.....	86
8.9. PROTOPLAST ATP DETERMINATION.....	87
9. RESULTS.....	88
9.1. SHOOT PHOTOSYNTHETIC CARBON ASSIMILATION.....	88
9.1.1. <u>Lycopersicon esculentum</u>	88
9.1.1.1. Pre-treatment effects.....	88
9.1.1.2. The influence of nitrate and ammonium.....	91
9.1.2. <u>Pisum sativum</u>	91
9.1.2.1. Pre-treatment effects.....	91
9.1.2.2. The influence of nitrate and ammonium.....	94
9.1.2.3. Carbon dioxide compensation points.....	98
9.1.3. Limitation in the use of the I.R.G.A.....	99
9.1.4. STOMATAL RESISTANCE.....	100
9.2. LEAF DISK PHOTOSYNTHETIC CARBON DIOXIDE ASSIMILATION.....	101
9.3. PROTOPLAST NITRATE, NITRITE AND AMMONIUM UPTAKE.....	105
9.3.1. Nitrate.....	105
9.3.2. Nitrite.....	106
9.3.3. Ammonium.....	109
9.4. PROTOPLAST CARBON DIOXIDE ASSIMILATION.....	111
9.4.1. Nitrate.....	112

9.4.2. Nitrite.....	112
9.4.3. Ammonium.....	115
9.5. PROTOPLAST OXYGEN EVOLUTION.....	116
9.5.1. Nitrate.....	116
9.5.2. Nitrite.....	116
9.5.3. Ammonium.....	118
9.5.4. Phosphate.....	118
9.5.5. Sodium bicarbonate.....	118
9.5.5.1. Interaction between Nitrate and Sodium Bicarbonate.....	121
9.5.5.2. Interaction between Ammonium and Sodium Bicarbonate.....	125
9.5.5.3. The effects of respiratory inhibitors.....	129
9.6. THE EFFECT OF NITRATE ON PROTOPLAST ATP LEVELS.....	133
10. DISCUSSION.....	135
10.1. SHOOT PHOTOSYNTHETIC CARBON ASSIMILATION.....	135
10.1.1. <u>Lycopersicon esculentum</u>	136
10.1.1.1. Pre-treatment effects.....	136
10.1.1.2. The influence of nitrate and ammonium.....	138
10.1.2. <u>Pisum sativum</u>	139
10.1.2.1. Pre-treatment effects.....	139
10.1.2.2. The influence of nitrate and ammonium.....	141
10.1.2.3. Carbon dioxide compensation points.....	142
10.2. STOMATAL RESISTANCE.....	145
10.3. LEAF DISK PHOTOSYNTHETIC CARBON DIOXIDE ASSIMILATION.....	147
10.4. PROTOPLAST NITRATE, NITRITE AND AMMONIUM NET UPTAKE.....	148
10.4.1. Nitrate.....	148
10.4.2. Nitrite.....	149
10.4.3. Ammonium.....	149
10.5. PROTOPLAST NET CARBON DIOXIDE ASSIMILATION.....	150
10.5.1. Nitrate.....	152
10.5.2. Nitrite.....	155

10.5.3. Ammonium.....	156
10.6. PROTOPLAST NET OXYGEN EVOLUTION.....	157
10.6.1. Nitrate.....	157
10.6.2. Nitrite.....	157
10.6.3. Ammonium.....	158
10.6.4. Phosphate.....	158
10.6.5. Sodium bicarbonate.....	159
10.6.5.1. Interaction between Nitrate and Sodium Bicarbonate.....	160
10.6.5.2. Interaction between Ammonium and Sodium Bicarbonate.....	161
10.6.5.3. The effects of respiratory inhibitors.....	162
10.7. EFFECT OF NITRATE ON PROTOPLAST ATP LEVELS....	163
11. CONCLUSION.....	164
12. REFERENCES.....	176

Appendices

A: Listing of Photosynthesis Program.....	183
---	-----

List of Tables

I: Table 1. Long Ashton Nutrient Medium.....	73
--	----

List of Figures

Fig. 1. A tentative model for NO_3^- uptake into Chara cells under different conditions of nitrate status. t = Tonoplast, m = plasma membrane, o = outside cell, i = inside cell, v = vacuole, a.a. = amino acids (Deane-Drummond, 1984).....13

Fig. 2. The C_4 dicarboxylic acid shuttle for the export of light generated reducing equivalents from illuminated chloroplasts and their utilization in the reduction of a

cytoplasmic substrates. Area within double lines represents chloroplast. Reaction I involves light coupled NADP-MDH with the concomitant evolution of O_2 . Reaction IIa involves the outward transport of malate with associated inward transport of OAA (reaction IIb) which is effected by the dicarboxylic acid translocator associated with the inner membrane. Reaction III is catalysed by NAD-MDH. Reaction IV can in theory be one of many reactions. Reaction IIc involves the inward transport of aspartate by the C_4 dicarboxylic acid translocator and transamination of OAA by chloroplast aspartate- α -KG amino transferase (reaction V) (After Anderson, 1981).....42

Fig. 3. The PGA/DHAP shuttle for the export of light-generated reducing equivalents and high-energy phosphate from illuminated chloroplasts. Reaction I to III involve enzymes of reductive CO_2 assimilation. DHAP is exported from the chloroplast (reaction IVA) with the counter transport of PGA (reaction IVB) via the phosphate translocator. The PGA and DHAP form part of the Calvin cycle intermediates pool. DHAP is oxidised in the cytoplasm in reactions involving enzymes of the glycolytic sequence (reaction V to VII) (After Anderson, 1981).....45

Fig. 4. Spectral analysis of light sources used for: a) Growth of plants; b) Gas exchange studies; c) Oxygen evolution studies.....72

Fig. 5. The gas circuit employed in the determination of photosynthetic CO_2 uptake using the ADC I.R.G.A.....75

Fig. 6. Comparison of effects of release from N starvation at variable times prior to determination of the photosynthetic rates of L. esculentum. Bars indicate S.E. where sufficient measurements were performed to calculate this statistic.....89

Fig. 7. The response of L. esculentum to 1 mM $NaNO_3$ supplied to plants released from N starvation at various times

prior to determination of photosynthetic rates. a) N supplied regularly (Normal), 0.4 and 1.6 days prior to measurement b) N supplied 2, 3 and 4 days prior to measurement. S.E. < 1.2.....90

Fig. 8. The effect of feeding various concentrations of NO_3^- to the bathing medium of cut normal N fed shoots of L. esculentum on the rate of photosynthetic carbon assimilation. a) Supply in range 0.1 mM - 0.3 mM b) Supply in range 1 mM - 10 mM. S.E. < 2.4.....92

Fig. 9. Summary of the response of L. esculentum shoot photosynthetic rate to NO_3^- . Rate % change was derived from the slope of the linear best-fit line of % change over time. Although all data points were retained, use of splines was made to derive shape of curve.....93

Fig. 10. The effect of feeding various concentrations (0.1 mM - 0.3 mM) of NH_4^+ to the bathing medium of cut shoots of L. esculentum on the rate of photosynthetic carbon assimilation. S.E. < 3.1.....93

Fig. 11. Photosynthetic rates of P. sativum either N starved or released from starvation at various times prior to measurement as compared with normally N fed plants. Effect of anaerobic conditions on photosynthetic rates of normally N fed plants included to illustrate importance of CO_2 disassimilation in determining photosynthetic rates. Photosynthetic rates expressed; a) on basis of leaf area b) on basis of chlorophyll content. Bars indicate S.E. where sufficient measurements were performed to calculate this statistic.....95

Fig. 12. The consequence of feeding various concentrations of NO_3^- to the bathing medium of cut shoots of P. sativum on the rate of photosynthetic carbon assimilation. a) Supply in range 0.3 mM - 0.5 mM. S.E. < 0.41. b) Supply in range 1 mM - 25 mM. S.E. < 1.1.....96

Fig. 13. The effect of feeding various concentrations (0.3 mM - 10 mM) of NH_4^+ to the bathing medium of cut shoots of P. sativum on the rate of photosynthetic carbon assimilation. S.E. < 1.3 for 0.3 mM and 1 mM NH_4^+ but S.E. < 15.6 for 10 mM.....97

Fig. 14. Summary of the response of P. sativum shoot photosynthetic rate to NO_3^- and NH_4^+ . Rate % change was derived from the slope of the linear best-fit line of % change over time. Bars indicate S.E.....97

Fig. 15. Compensation points of P. sativum either N starved or released from starvation at various times prior to measurement as compared with normally N fed plants. Effect of anaerobic conditions on compensation points of normally N fed plants included to illustrate the dependence CO_2 dissimilation on the presence of O_2 . Bars indicate S.E. where sufficient measurements were performed to calculate this statistic.....98

Fig. 16. The effect of excising the root from the pea plant on the stomatal resistance of the shoot. Bars indicate S.E.100

Fig. 17. Stomatal resistance changes in response to the introduction of 10 mM concentrations of various salts into the bathing medium of cut shoots. NO_3^- supplied as NaNO_3 and NH_4^+ as NH_4Cl . Control comprised of plants left in distilled water. Measurements performed 90 minutes after cutting. a) Data normalised around mean starting value. b) Normalised data with NaCl treatment assumed as a reference102

Fig. 18. Stomatal resistance changes in response to the introduction of 20 mM concentrations of various salts into the bathing medium of cut shoots. NO_3^- supplied as NaNO_3 and NH_4^+ as NH_4Cl . Control comprised of plants left in distilled water. Measurements performed 90 minutes after cutting. a) Data normalised around mean starting value.

b) Normalised data with NaCl treatment assumed as a reference,	103
Fig. 19. The influence of NO_3^- on <u>P. sativum</u> leaf disc photosynthetic $^{14}\text{CO}_2$ assimilation at various times after infiltration. Bars indicate S.E.	104
Fig. 20. Nitrate reductase production of NO_2^- in protoplasts under dark anaerobic conditions. Bars indicate S.E.	106
Fig. 21. The uptake of NO_2^- at different exogenous concentrations of this ion over time	107
Fig. 22. The concentration dependence of NO_2^- uptake by protoplasts after different times of exposure to NO_2^- .	108
Fig. 23. The kinetics of NO_2^- uptake after 10 minutes of exposure to NO_2^- . The K_m determined from a Lineweaver-Burk plot of this data was 0.62 mM. Bars indicate S.E.	108
Fig. 24. The uptake of NH_4^+ different exogenous concentration of this ion over time	109
Fig. 25. The concentration dependence of NH_4^+ uptake by protoplasts after different times of exposure to NH_4^+ .	110
Fig. 26. The kinetics of NH_4^+ uptake after 10 minutes of exposure to NH_4^+ . The K_m determined from a Lineweaver-Burk plot of this data was 0.039 mM if last three data points were neglected. Bars indicate S.E.	111
Fig. 27. Response of protoplast CO_2 assimilation to the supply of nitrate at a) high light intensity ($600 \mu\text{E.m}^{-2}.\text{s}^{-1}$) b) low light intensity ($200 \mu\text{E.m}^{-2}.\text{s}^{-1}$). Bars indicate S.E.	113

Fig. 28. Comparison of the response of protoplast CO_2 assimilation to the supply of nitrate at high and low light intensities (600 and $200 \mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$). Bars indicate S.E.114

Fig. 29. Raw data (low light intensity) showing the response of protoplast $^{14}\text{CO}_2$ assimilation to the supply of NO_3^- at various concentrations. Initial rates are taken as reference point (0%).....114

Fig. 30. Response of protoplast CO_2 assimilation to the supply of nitrite. Bars indicate S.E. where larger than symbols.....115

Fig. 31. Response of protoplast CO_2 assimilation to the supply of ammonium. Bars indicate S.E. where larger than symbols.....115

Fig. 32. The responses of pea protoplasts O_2 evolution capacity to the supply of 1.6 mM of three salts (NaCl , NaNO_3 and NH_4SO_4). The initial slopes are portrayed as starting from -4 minutes to 0 minutes when the various salts were added. The broken lines represent the null hypothesis i.e. no effect of the salts.....117

Fig. 33. The influence of NO_3^- concentration on the evolution of O_2 from protoplasts expressed as percent increase above the initial rate prior to the addition of NO_3^- . All values indicated passed a significance test at the $p=0.05$ level. Bars indicate S.E.....117

Fig. 34. The influence of NO_2^- concentration on the evolution of O_2 from protoplasts expressed as percent increase above the initial rate prior to the addition of NO_2^- . All values indicated passed a significance test at the $p=0.05$ level. Bars indicate S.E.....119

Fig. 35. The influence of NH_4^+ concentration on the evolution of O_2 from protoplasts expressed as percent increase

above the initial rate prior to the addition of NH_4^+ . All values indicated passed a significance test at the $p=0.05$ level. Bars indicate S.E.....120

Fig. 36. The influence of PO_4^{2+} concentration on the evolution of O_2 from protoplasts expressed as percent increase above the initial rate prior to the addition of PO_4^{2+} . All values indicated passed a significance test at the $p=0.05$ level. Insufficient replication was done to allow calculation of S.E.....120

Fig. 37. Changes in oxygen evolution in response to sequential additions of NaHCO_3 . a) and b) Concentration O_2 in protoplast suspension. c) Rate O_2 evolution as influenced by additions.....120a

Fig. 38. Changes in oxygen evolution by protoplasts incubated without NaHCO_3 in response to different concentrations of NO_3^- . a) O_2 concentration in protoplasts suspension. b) Rate of O_2 evolution as influenced by additions.....122

Fig. 39. Changes in oxygen evolution by protoplasts incubated in 1 mM NaHCO_3 in response to different concentrations of NO_3^- . a) O_2 concentration in protoplasts suspension. b) Rate of O_2 evolution as influenced by additions.....123

Fig. 40. Changes in oxygen evolution by protoplasts incubated in 5 mM NaHCO_3 in response to different concentrations of NO_3^- . a) O_2 concentration in protoplasts suspension. b) Rate of O_2 evolution as influenced by additions.....124

Fig. 41. Changes in oxygen evolution by protoplasts incubated in 1 mM NaHCO_3 in response to different concentrations of NH_4^+ . a) O_2 concentration in protoplasts suspension. b) Rate of O_2 evolution as influenced by additions.....126

Fig. 42. Changes in oxygen evolution by protoplasts incubated in 5 mM NaHCO_3 in response to different concentrations of NH_4^+ . a) O_2 concentration in protoplasts suspension. b) Rate of O_2 evolution as influenced by additions127

Fig. 43. Changes in oxygen evolution by protoplasts incubated in 10 mM NaHCO_3 in response to different concentrations of NH_4^+ . a) O_2 concentration in protoplasts suspension. b) Rate of O_2 evolution as influenced by additions128

Fig. 44. Changes in oxygen evolution by protoplasts incubated in 10 mM NaHCO_3 in response to different concentrations of MFA and KCN. a) O_2 concentration in protoplasts suspension. b) Rate of O_2 evolution as influenced by additions130

Fig. 45. Changes in oxygen evolution by protoplasts in response to different concentrations of NaHCO_3 and NO_3^- . a) O_2 concentration in protoplasts suspension incubated in 1 mM MFA. b) a) O_2 concentration in protoplasts suspension not incubated in MFA. c) i) Rate of O_2 evolution by protoplasts incubated in 1 mM MFA as influenced by additions ii) i) Rate of O_2 evolution by protoplasts not incubated in MFA as influenced by additions.....131 - 132

Fig. 46. Changes in oxygen evolution by protoplasts incubated in 1 mM MFA and 10 mM NaHCO_3 in response to different concentrations of NO_3^- . a) O_2 concentration in protoplasts suspension. b) Rate of O_2 evolution as influenced by additions.....133

Fig. 47. Response of ATP levels in protoplasts to the supply of NO_3^- at two concentrations. Low concentration (0.17 mM) selected for its stimulatory effect while 1 mM exhibited an inhibitory effect on CO_2 evolution. Percentage change calculated as deviation from the control....134

Fig. 48. The pathways suggested to account for the effects of NO_3^- , NO_2^- , NH_4^+ and PO_4^{2+} effects on the photosynthetic CO_2 assimilation and O_2 evolution. Positive effectors (+); negative effectors (-). The key enzymes are numbered. (1). ADPglucose pyrophosphorylase; (2). phosphorylase; (3). fructose-1,6-bisphosphatase; (4). sucrose phosphate synthase; (5). 2-phosphofructokinase; (6). hexokinase; (7). phosphofructokinase; (8). PEP carboxylase; (9). pyruvate kinase; (9). nitrate reductase (After Elrifi and Turpin, 1987).....166

6. INTRODUCTION

Light, temperature, water availability and mineral nutrients are the major environmental factors which determine plant productivity (Yoneyama, 1984). Availability of nitrogen limits growth in many ecosystems. In agriculture, mineral nutrition is routinely manipulated to increase yield and crop productivity. Supply of nitrogen strongly affects plant growth and dry matter productivity which are related to photosynthetic carbon assimilation (Cresswell, 1980). It is well known that nitrogen supply can change crop photosynthetic rates by affecting leaf areas or photosynthetic rates per leaf area (Yoneyama, 1984). The mechanisms through which these effects occur are complex and multifaceted. Conflicting reports exist regarding the effect of inorganic nitrogen on photosynthetic carbon assimilation. Some of the conflict at least arises out of different experimental conditions and ways of expressing data.

A large number of physiological parameters have been found to be influenced by the supply of NO_3^- . In Amaranthus powellii total leaf N, chlorophyll content, specific leaf mass, leaf area, relative growth rate, unit leaf rate and net production rate decreased with decreasing NO_3^- concentration while the leaf area ratio increased (Hunt et al., 1985a). In addition stomatal conductance was sensitive to the vapour pressure difference between leaf and air in plants with high total leaf nitrogen concentrations while plants with low total leaf nitrogen showed relative insensitivity (Hunt et al., 1985b). This indicates the possibility of improved water usage in plants supplied with nitrogen. A number of photosynthetic parameters (assimilation rate at 340 $\mu\text{bar CO}_2/\text{bar}$, stomatal conductance, CO_2 and light saturated net photosynthesis, RuBPC activity and PEPc activity) were found to be positively related in a linear fashion to leaf NO_3^- content (Hunt et al., 1985c).

Frank and Marek (1983) determined that the carbon compensation points of barley were increased by N deficiency while photosynthetic rates were decreased. This was accompanied by an increase in mitochondrial respiration under N deficient conditions (Marek, 1984). This was concluded to imply that the change in compensation points was attributable to the alteration in mitochondrial respiration (Marek and Frank, 1984). There are numerous other reports of the effects of NO_3^- and NH_4^+ on photosynthetic and photorespiratory activity. Vaklinova et al., (1981) report that NO_3^- and NH_4^+ ions enhance photosynthetic and photorespiratory activity in pea protoplasts and barley leaves. The form and concentration of nutrient medium nitrogen has been reported to influence the net photosynthetic rate and CO_2 compensation points of C_3 and C_4 grasses (Tew et al., 1974; Cresswell et al., 1977). Vacuum infiltration of nitrate and ammonium ions into nitrogen starved leaves of Themeda triandra and Zea mays increased CO_2 compensation points and nitrate increased net photosynthesis but ammonium ions inhibited net photosynthesis (Amory and Cresswell, 1984). Evidence was advanced by these authors suggesting that an alternative pathway for photorespiration may exist involving formate and suggested that this pathway may become effective at high levels of N fertilization (Amory and Cresswell, 1986). In contrast Stepanova et al. (1980) found a reduction of CO_2 assimilation and indications of an increase in photorespiratory activity in maize supplied with nitrate. Change of the nitrogen growth regime from NO_3^- to NH_4^+ has been reported to result in no change in CO_2 assimilation or photorespiratory activity (Hall et al., 1984; Klaus et al., 1985).

Although inorganic nitrogen has been suggested to regulate metabolism through a number of specific effects, no consensus of opinion exists. This investigation was undertaken in an attempt to gain clarity on the influence of inorganic nitrogen on photosynthetic carbon assimilation and the possible mechanisms of any effects. As a result of the rapid metabolism of inorganic nitrogen, the primary aim of this study was to ascertain the short term effects of inorganic nitrogen.

7. LITERATURE REVIEW

7.1. TRANSPORT AND TRANSLOCATION OF NITROGEN

Like many metabolic reactions, the reduction of nitrogen requires the movement of nitrogenous compounds between cellular compartments. A number of metabolic processes involve extensive membrane transport. The reduction of NO_3^- is thought to be extrachloroplastic while NO_2^- reduction and NH_4^+ assimilation to amino acids is chloroplastic. The photorespiratory cycle involves the movement of amino acids between the chloroplast, peroxisome and the mitochondria. Ammonium released in the process in the mitochondrion is probably reassimilated in the cytoplasm and chloroplast. Thus component parts of some metabolic pathways involve highly specific and often large volume translocation across a membrane. Each membrane system has its own unique characteristics which place different restraints on transport. The chloroplast and mitochondrial outer and inner membranes are, for instance, substantially different, exhibiting different protein and lipid compositions and permeability characteristics.

Nitrogen metabolism is well known to be closely integrated with carbon assimilation. This involvement extends to the interrelationships between the movements of nitrogen and carbon across membranes. The transport of nitrogen compounds may be through active or passive mechanisms. Even if the proposed active mechanisms do not directly involve carbon metabolism, energy coupling invariably involves the withdrawal of energy from a common pool resulting in secondary effects on carbon metabolism.

The purpose of this review is to discuss the mechanisms, both

physical and physiological, pertaining to the intracellular transport and assimilation of nitrogen. Much of the work referred to is with algae, fungi or bacteria because the higher plant systems have been incompletely studied.

7.1.1. PLANT ROOT UPTAKE OF INORGANIC NITROGEN

The amount and form of nitrogen in soils depends on the extent of mineralization of the soil and nitrification. Ammonium may be tightly held by the micaceous clay minerals of the soil. Plant roots in soils absorb N largely as NO_3^- since this ionic form occurs in higher concentration than NO_2^- and NH_4^+ and is free to move within the root solution (Reisenauer, 1978). This has the consequence that it may thus be readily lost through leaching. Some NH_4^+ is usually present and may affect growth to some extent. Ammonium requires less energy for its reduction than NO_3^- and it may thus be supposed that it would be the preferred species for uptake although from evidence derived from root carbohydrates and growth it appears that ammonium may require more energy than nitrate (Reisenauer, 1978). Increasingly nitrification inhibitors are being applied in agriculture to elevate the levels of soil NH_4^+ . On the other hand NH_4^+ may exhibit some toxic effects (reduced photosynthesis and respiration, susceptibility to water stress) on plants. The uptake systems for NO_3^- and NH_4^+ are separate and are affected differently by pH, temperature and carbohydrate supply (Haynes and Goh, 1978). Nitrite uptake by plant roots is not generally considered to be of consequence as a result of the low levels of NO_2^- in the soil and the reported toxicity of this ion. Nitrite may arise in the soil from transformation of nitrogen compounds in the soil and rhizosphere, from organic wastes or from NO_3^- containing roots during oxygen stress (Bretler and Luczak, 1982).

The kinetics of NO_3^- uptake are unlike those of other ions; instead of a constant rate of absorption, NO_3^- uptake exhibits an early lag phase followed by a more rapid phase of absorption (Ashley et al., 1975; Haynes and Goh, 1978). The duration of this induction is reduced by higher NO_3^- concentrations (Morgan et

al., 1985). Uptake and reduction of NO_3^- are often similar indicating at least some inter-dependence and similar regulation of these two systems. Evidence that these two systems (uptake and reduction) are not identical is presented later. Uptake of NO_3^- is saturated with external concentrations around 1 mM for most plants (Dale, 1979) although this is subject to considerable variation. Plants can absorb NO_3^- during both the light and dark portions of the daily cycle at nearly equal rates (Rufy et al., 1984). The NO_3^- accumulated in the dark period is however only translocated and reduced, predominantly in the shoot (80% in soybean), in the following light period (Rufy et al., 1984). Root absorption of NO_3^- may result in the passage of the absorbed ions into:

- xylem vessels
- root medium
- reduction via NR and NiR to NH_4^+
- deposition in root vacuoles which probably accounts for most of the tissue NO_3^- (Jackson et al., 1986).

The pathway of NO_3^- into the root is likely to be strongly influenced by the position of NR within the root. NR has been reported to occur predominantly in the epidermal cells of the maize root (Rufy et al., 1984). This observation is consistent with observation reported below of limited reduction of endogenous NO_3^- . Of net influx during 24 hours exposure to 1 mM K^{15}NO_3 , 56% was translocated, 30% reduced and 14% accumulated in maize roots (Jackson et al., 1986). The site at which NO_3^- is reduced varies between plant species. In some reduction is predominantly in the root and in others predominantly in the shoot. In rice it appears from ^{15}N studies that nitrate is largely incorporated into glutamine and glutamic acids in the root (Yoneyama and Kumazawa, 1975). In contrast shoot reduction has been observed for barley by Lewis et al. (1983). The proportion of NO_3^- translocated is dependent to some extent on the concentration supplied. At greater NO_3^- concentrations more NO_3^- was translocated with the percentage NO_3^- reduced in the root remaining relatively constant, but the amount of translocated reduced N increased from 10% to 50% (Morgan et al., 1985a;b). The amino

acid products of NO_3^- reduction and NH_4^+ assimilation may be accumulated in vacuoles, translocated to the xylem or utilised in protein synthesis, the latter consuming a fairly constant proportion of NO_3^- (25-29%) (Jackson et al., 1986). A NO_3^- uptake uniporter and efflux nitrate-proton symporter is thought to exist (Deane-Drummond, 1984c). Maize roots, bathed in deionized water, were found to excrete H^+ actively without concomitant cation uptake (Mengel and Schubert, 1985). A NO_3^- insensitive H^+ ATPase has been identified in maize roots by O'Neill et al. (1983). Thus the ATPase for proton export is available in the maize root and may be coupled to NO_3^- efflux. Reduction of NO_3^- results in the production of OH^- (Raven and Smith, 1976) which may in turn participate in an antiport for NO_3^- uptake. Thus any decrease in NO_3^- reduction may also influence uptake to some extent although NO_3^- uptake is not obligatorily coupled to reduction (Jackson et al., 1986). MacKown et al. (1983) report little reduction but appreciable translocation and efflux of NO_3^- during the influx of this ion, while extensive reduction, but little translocation and efflux occurred, in the absence of influx. The uptake into roots may be thought of as a three compartment system with the cytoplasm functioning both as a destination for NO_3^- from the root solution and the vacuole (storage pool) and as a source for vacuole, xylem and root solution NO_3^- (MacKown et al., 1983; Jackson et al., 1986). The NO_3^- in the vacuole does not however appear to be reduced when sufficient exogenous NO_3^- is available but is reduced in the absence of exogenous NO_3^- (Jackson et al., 1986).

Uptake of NO_3^- into plant roots is the net result of both influx and efflux in what may be considered to be a futile cycle. Total efflux may represent as much as 50% of influx (Jackson et al., 1986). The apparent induction of NO_3^- uptake has been observed in a number of plants and has been ascribed to NO_3^- stimulated synthesis of the uptake system (from protein synthesis inhibitor evidence) although changes in the efflux system could also be a factor in modifying uptake (Jackson et al., 1986).

Plants absorb ions from a multi-ion environment and the interactions between these ions are often complex. The effects of one ion

on the uptake of another are important when considering the combined effects of inorganic nitrogen species. For example, the report that NO_3^- reduced $^{14}\text{CO}_2$ accumulation by maize while the combination of NO_3^- and NH_4^+ increased ^{14}C accumulation (Morot-Gaudry et al., 1985) may be related to uptake phenomena. Supply of NH_4^+ has been reported to both stimulate and retard NO_3^- and K^+ uptake by barley but these effects were strongly dependent on the cultivar of barley used and the accompanying ions supplied with the NH_4^+ (Bloom and Finazzo, 1986). The absorption of NO_3^- decreased the capacity of the plant for anion uptake and increased the capacity for cation uptake, presumably as a result of charge balance requirements (Haynes and Goh, 1978). Lewis et al. (1983) found that nitrogen supplied as NO_3^- to barley was predominantly assimilated in the shoot (66% xylem sap NO_3^-) while NH_4^+ was assimilated in the root (93% xylem sap organic N). With a combination of NO_3^- and NH_4^+ feeding only 29 % of the xylem sap was in the form of nitrate, indicating a suppression of NO_3^- absorption by NH_4^+ . Reduced net NO_3^- uptake in the presence of NH_4^+ has been reported for numerous higher plants although little or no effects have been evident in others (Haynes and Goh, 1978; MacKown et al., 1982). This effect is apparently not exclusively reduction related, because influx is more strongly inhibited by NH_4^+ than NO_3^- reduction (Morgan et al., 1985b). A number of mechanisms were suggested by MacKown et al. (1982) to account for these effects:

- enhanced efflux of NO_3^-
- allosteric inhibition of the uptake system
- inactivation or repression of NR activity thereby influencing uptake
- decreased availability of carbon substrates and ATP
- alteration in net synthesis or regulation of uptake system.

Supply of NH_4^+ to maize did not alter the levels of tissue NO_3^- and thus efflux was not apparently enhanced by this treatment. Ammonium is well known to act as an uncoupler of non-cyclic photophosphorylation resulting in a rise in reducing potential and ADP which may result in the inactivation of NR (Haynes and

Goh, 1978). Precisely how this uncoupling (presumably chloroplastic) results in an increase in cytoplasmic reducing potential and ADP is not clear. From the differential inhibition of uptake and reduction it was suggested that the uptake system was itself influenced although ME_4^+ and its reduction products have been suggested by other workers to affect uptake by altering NR activity (MacKown et al., 1982). Although the net uptake of NO_3^- was smaller (MacKown et al., 1982; Lewis et al., 1983), accumulation of NO_3^- in roots was enhanced by application of NH_4^+ while the accumulation of reduced N was inhibited (MacKown et al., 1982).

The absorption of NH_4^+ by plant roots is rapid but is subject to feedback controls, the most important of which is pH (Reisenauer, 1978). The absorption of NH_4^+ increased with pH which may be related to the NH_4^+ concentration (Reisenauer, 1978). In addition the uptake of the NH_4^+ cation results in the accumulation of cations within the plant in contrast to the accumulation of anions as a result of NO_3^- uptake. The balance may be restored through the synthesis and decarboxylation of organic acids. Ammonium absorbed by the roots is, in virtually all recorded cases, converted through reductive amination of α -ketoglutarate to glutamate and glutamine prior to translocation, although in peas asparagine may play an important rôle as the major translocated amino acid form (Ta and Joy, 1984). Absorption of divalent anions (PO_4^{2-} , SO_4^{2-}) was increased while absorption of divalent cations was decreased by NH_4^+ (Reisenauer, 1978; Haynes and Goh, 1978). The effects of NH_4^+ on uptake of monovalent ions were inconsistent (Reisenauer, 1978).

Uptake of 0.1 mM NO_2^- was found to occur in roots of Phaseolus vulgaris against an electrochemical gradient. The rate of uptake exhibited an inverse relationship to medium NO_2^- concentration (Bretler and Luczak, 1982). At pH 5, 2% of NO_2^- is in the form of nitrous acid which is believed to be the toxic form of NO_2^- and this may account for reduced uptake. The uptake of NO_2^- may affect other aspects of metabolism or it could restrict its own uptake. Of the absorbed NO_2^- only one third accumulated as NO_2^- in the root (Bretler and Luczak, 1982). Although NO_2^- can induce NR,

it does not appear to be capable of inducing NO_3^- uptake (Bretler and Luczak, 1982).

For the majority of plants the best growth and highest protein production rates are achieved with a mixture of NH_4^+ and NO_3^- in the proportion 30 mM:10 mM (Reisenauer, 1978). Maximum rates of growth and N assimilation were reported by Lewis et al. (1983) to occur in barley maintained on a mixture of NO_3^- and NH_4^+ . The optimum depends on the species of plant, age and pH of growth medium (Haynes and Goh, 1978). Root temperature has been indicated to play an important role in determining the specific absorption rates for NH_4^+ and NO_3^- although the overall N-intake remained relatively constant (Clarkson et al., 1986).

7.1.2. Transport of nitrogen compounds across the plasmalemma

7.1.2.1. Nitrate transport

Although one might expect the plasmalemma to play an important role in the regulation of nitrogen flux into the cell, it appears that this control is predominantly exerted at the tonoplast and any control at the plasmalemma is simply a 'pump and leak' system (Glass et al., 1985). NO_3^- 'leakage' across the plasmalemma down the electrochemical gradient, which can be stimulated by NH_4^+ , may be appreciable in higher plant roots and may be important in determining the overall rate of translocation (Deane-Drummond, 1985). In Chara a linear relationship between NO_3^- efflux and cytoplasmic $[\text{NO}_3^-]$ was found to exist. This linear relationship may be modified by the addition of NH_4^+ (Deane-Drummond, 1985). Efflux was concluded to be active because the electrochemical driving force is in favour of NO_3^- entry, which is not to say that NO_3^- entry is passive, but that it may be accelerated by a carrier (Deane-Drummond, 1985). If cytoplasmic NO_3^- is very high then influx is active and efflux passive (Deane-Drummond, 1985).

The site of proton linked NO_3^- reduction/transport in spheroplasts prepared from Escherichia coli has been shown,

through the use of azide inhibition, to be on the outer aspect of the cytoplasmic membrane, while the FMN₂ reducible site is on the inner aspect (Garland et al., 1975). With malate supplied as a source of reductant, a ratio of 4 H⁺/NO₃⁻ (H⁺ efflux:NO₃⁻ supply) was found while formate and succinate yielded a ratio of 2. The rate of entry of NO₃⁻ into spheroplasts by passive diffusion was found to account for 0.1% of the NO₃⁻ required for NO₃⁻ reduction (Garland et al., 1975). In haeme deficient mutants, no uptake of NO₃⁻ occurred indicating a link between respiratory electron transport and NO₃⁻ reduction/transport. In addition lipophilic carriers facilitating transport of cations (K⁺ - valinomycin and H⁺ - CCCP) stimulated transport of NO₃⁻ across the membrane indicating the electrogenic nature of NO₃⁻ transport. This has led to the proposal that NO₃⁻ reduction is vectorial utilizing reducing potential from the cytoplasm for the reduction and transport of NO₃⁻ into the cytoplasm (Garland et al., 1975).

Active transport mechanisms may be divided into two classes:

- 1) carrier mediated - carrier molecule facilitating membrane transport with the requirement for an energy supply
- 2) vesicle mediated - vesicular exocytosis or endocytosis.

Vesicle mediated transport has received some support recently (Selga, 1983) and may provide for nonspecific transport. Carrier mediated transport may involve a number of different mechanisms of energy coupling:

- 1) group translocation
- 2) primary active transport
- 3) coupled flow.

Butz and Jackson (1977) suggest that the transport and reduction of NO₃⁻ is a group translocation type of transport. They envisioned a NR tetramer associated with an ATPase in the plasmalemma capable of binding NAD(P)H and functioning in both the transport and reduction of NO₃⁻ in a 3:1 ratio. A similar dimer system was proposed for the chloroplast envelope functioning in a 1:1 transport:reduction. These authors presented correlative evidence

that NR activity is coupled to transport of nitrate. Correlative evidence for this dual rôle may be drawn from the finding that the electrophoretic properties of NR differ between diatoms (clones of Biddulphia aurita) living in different temperature zones and capable of different NO_3^- uptake rates at various temperatures (Underhill, 1977). Evidence of this nature is not acceptable however, in that some of the algae have no vacuoles and thus lack a substantial storage capacity. The rate of uptake in this case should be determined by the rate of reduction purely as a consequence of the unlikely event of large amounts of NO_3^- accumulating in the cytoplasm. In addition mutants of Arabidopsis thaliana exhibiting no NR activity were capable of significant rates of NO_3^- uptake (Doddema et al., 1978). The rôle of NR in transport of NO_3^- has largely been discredited due to a lack of evidence and the dependence of uptake on reduction in the non-vacuolate systems described above.

The involvement of an ATPase in NO_3^- uptake was proposed because of the sensitivity of some ATPases to NO_3^- and the apparent sensitivity of NO_3^- reduction to adenylates. The ATPase was suggested to play a rôle in transporting the NO_3^- to reducing sites across the membrane. Alternatively the ATPase was suggested to facilitate the coupling of a transmembrane proton gradient with ATP formation and thus NO_3^- reduction (Butz and Jackson, 1977). This proposal is however lacking in experimental evidence. The only NO_3^- sensitive ATPases are those found in the tonoplast, and thus it is unlikely that the regulation of the ATPase on the plasmalemma can be achieved by nitrate. Although doubt exists about the rôle of ATPase in NO_3^- transport, evidence is available for the existence of permeases across the plasmalemma (Fuggi, 1985).

A substantial amount of evidence exists for the implication of a $\text{NO}_3^-/\text{OH}^-$ counter transport (or NO_3^-/H^+ co-transport) in NO_3^- uptake. An immediate transient depolarization, followed by repolarization, of the membrane potential in Lemna gibba was found on addition of 20 mM NO_3^- (Novacky and Fischer, 1978). This application is however extremely concentrated and not in the physiological range of NO_3^- levels at all. Later work by Ullrich

and Novacky (1981) showed that smaller concentrations (0.2 mM) had a similar effect. In addition these authors showed that plants with low membrane potentials showed no depolarization and even a slight hyperpolarization on supply of light (Ullrich and Novacky, 1981). Two permease systems (high and low affinity) have been proposed for NO_3^- uptake into Cyanidium caldarium. The high affinity uptake system co-transported 1 NO_3^- and 2 H^+ with the NO_3^- binding first to the transport system (Fuggi, 1985). Although light is known to cause a reduction in the extent of membrane depolarization on supply of NO_3^- over dark conditions, it is thought that this light induced decrease in the depolarization on supply of NO_3^- is due to the photosynthetically linked simultaneous extrusion of protons from the cells. Thus it appears that the NO_3^- uptake process involves a H^+/NO_3^- co-transport (Novacky et al., 1978).

Correlative evidence may be found from the similarities of NO_3^- and pH changes to changes in the membrane potential (Ullrich and Novacky, 1981). If this type of system is operative for NO_3^- uptake in a 1:1 stoichiometry, then the uptake of NO_3^- is an electrically neutral process (Deane-Drummond, 1984c). Ammonia and NO_3^- utilization is reported to be accompanied by a decrease and increase in the external pH respectively (Fuggi et al., 1981). This alkalization during uptake and metabolism of NO_3^- has been reported to proceed with the stoichiometric release of 1 or 2 H^+ , depending on whether or not NO_3^- derived NH_4^+ was incorporated or not (Eisele and Ullrich, 1977; Fuggi et al., 1981; Novacky et al., 1978). Under steady state conditions, the incorporation of each equivalent of ammonia was accompanied by 0.8-1 equiv. of protons while for each equiv. of NO_3^- 1-1.2 equiv. protons were absorbed (Fuggi et al., 1981). Immediate alkalization has been reported during NO_3^- uptake as well as acidification although the alkalization has been disputed to have any rôle in NO_3^- uptake.

Additional supporting evidence comes from the report that $^{36}\text{ClO}_3^-/\text{NO}_3^-$ flux into Chara was to be sensitive to pH and exhibit a maximum at pH 4.5 (Deane-Drummond, 1984b).

The effect of pH on NO_3^- uptake has been reported to depend on the concentration of NO_3^- supplied, with greater pH dependence at lower NO_3^- concentrations and with maximal uptake occurring at pH 4.7 (Deane-Drummond, 1984c). This author found no correlation between alkalization and NO_3^- uptake. The proton pump inhibitor, DES, was found to dramatically stimulate NO_3^- uptake, although extremely high concentrations of DES had an inhibitory effect (Deane-Drummond, 1984c). An effect of NH_4^+ on the uptake of NO_3^- has been reported for Penicillium chrysogenum although in this case glutamine and asparagine supply was reported to result in a similar response (Goldsmith et al., 1973). The effect of NH_4^+ , or a metabolic product thereof was proposed to occur through one of the four mechanisms:

- a) It may promote the inactivation of NR
- b) It may act as an allosteric inhibitor of the NO_3^- uptake system
- c) A NH_4^+ utilizing system (enzyme) may inactivate NR
- d) It may repress the synthesis of NO_3^- uptake associated proteins.

Ammonia has been shown to have a similar effect to DES and the evidence pointed to a stimulation of NO_3^- efflux by NH_4^+ as the mechanism responsible for reduced NO_3^- uptake (Deane-Drummond, 1984b).

This effect was explained as being the result of an inhibitory effect on the efflux of NO_3^- from the cells and NO_3^- efflux was proposed to occur in exchange for NO_3^- influx. Nitrate efflux has previously been reported to be an important component of net NO_3^- uptake and to be independent of the supply of DES (Deane-Drummond, 1984a). In kinetic studies it was found that the uptake of $^{36}\text{ClO}_3^-$ was inhibited competitively by NO_3^- at low concentrations while at higher concentrations inhibition was not characteristic of competitive or non-competitive inhibition (Deane-Drummond, 1984a). These results have led to the following conclusions:

- a) NO_3^- efflux is not a simple electrochemical diffusion

- b) NO_3^- efflux and NO_3^- influx are linked
- c) Protons have a direct effect on the activity of the NO_3^- transporter.

A model proposed by Deane-Drummond (1984c) and Deane-Drummond (1984a) (Fig. 1) envisages a counter exchange, influenced by protons, of NO_3^- and $\text{HCO}_3^-/\text{OH}^-$ or $\text{HCO}_3^-/\text{H}^+$ at low external NO_3^- concentrations while at higher concentrations, the external NO_3^- may exchange for internal NO_3^- where net NO_3^- uptake is determined by reduction and transport into the vacuole. Although a single carrier has been proposed for both influx and efflux of NO_3^- , Deane-Drummond (1985) has found evidence for the existence of two independent carriers. The differential effects of inhibitors (DES inhibited efflux and NH_4^+ stimulated efflux) on efflux and influx and apparent independence of NO_3^- efflux of external $[\text{NO}_3^-]$ have been taken as evidence for two carriers. The influx of NO_3^- is thought to occur in exchange for HCO_3^- . The control of NO_3^- efflux from the cell is proposed to occur to prevent the unnecessary reduction of NO_3^- when alternative more reduced, and thus, less expensive forms of nitrogen are available (Deane-Drummond, 1985).

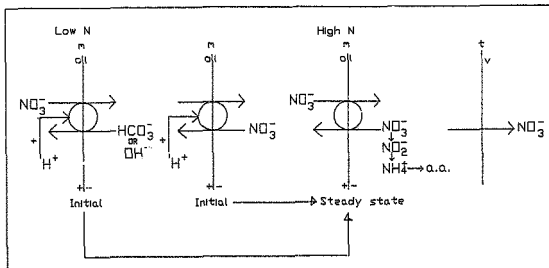


Fig. 1. A tentative model for NO_3^- uptake into *Chara* cells under different conditions of nitrate status. t = tonoplast, m = plasma membrane, o = outside cell, i = inside cell, v = vacuole, a.a. = amino acids (Deane-Drummond, 1984).

7.1.2.2. Ammonium uptake

Nonspecific diffusion of NH_4^+ across membranes has been favoured by some authors, but evidence for carrier mediated NH_4^+ transport has accumulated recently (Bertl et al., 1984). Addition of NH_4^+ has been reported to cause a concentration dependent change in cellular pH in *Riccia fluitans* (Bertl et al., 1984). On addition of $1 \mu\text{M}$ NH_4Cl the cytoplasmic pH of 7.2-7.4 dropped by 0.1-0.2 pH units, but shifted to pH 7.8 in the presence of $50 \mu\text{M}$ NH_4Cl . The vacuolar pH increased drastically from 4.5 to 5.7 with $50 \mu\text{M}$ NH_4Cl . These results were interpreted by the authors to imply the operation of two processes: first, acidification due to deprotonation of NH_4^+ ; and second alkalization through protonation of NH_3 which is taken up to a significant extent from high external concentrations. Recently an energy dependent specific

uptake of NH_4^+ by both a high (pH optimum 7.4) and a low (pH optimum 5.2) affinity system has been described for Anacystis nidulans. Both transport systems were found to be light dependent and sensitive to DCMU (Kashyap and Singh, 1985).

7.1.2.3. Amino acid transport

The transport of amino acids is accompanied by a simultaneous proton transport, although no alkalization of the external medium during steady state uptake was found (Novacky et al., 1978). A large variety of cells have been reported to possess a transport system for amino acids. In Chlorella vulgaris the transport system has been shown to be inducible by the supply of hexoses and by nitrogen shortage (Sauer et al., 1983). This induction was attributed to the C/N ratio. An amino acid transport system in Chlorella vulgaris has also been described which is inducible with NH_4^+ and NO_3^- when supplied with glucose (Sauer, 1984). The more rapid utilization of amino acids than of NO_3^- by sugarcane suspension cultures has led to the suggestion of a specific transport system for amino acids. Several translocators have been proposed for either specific amino acids or groups of amino acids (Robinson and Beevers, 1981; Felle, 1981). The uptake of amino acids has been proposed to be related to the influx of protons across the plasmalemma. An immediate depolarization of the plasmalemma membrane potential in Lemna gibba was induced through the addition of glycine. This depolarization suggested that glycine transport is coupled to a H^+ extrusion mechanism, probably maintained by an ATPase, and that amino acid transport occurs with the concomitant uptake of 1 H^+ (Novacky et al., 1978). Similar results were obtained using sugarcane tissue sections (Franz and Tattar, 1981) and oat coleoptiles (Kinraide and Etherton, 1980).

In oat coleoptiles strong pH insensitive depolarization of membrane potentials by basic amino acids and pH sensitive membrane depolarization by acidic and neutral amino acids has been reported. This led to the proposal by Kinraide and Etherton (1980) that neutral amino acids are co-transported with a H^+ and

that acidic amino acid transport is associated with a cation and a H^+ , while basic amino acid transport is not associated with proton co-transport. Sugarcane suspension cells have been shown to have three amino acid uptake systems specific for the net charge on the amino acid. Uptake of neutral amino acids is coupled with the uptake of 1 H^+ /amino acid and the efflux of 1 K^+ , while basic amino acids are imported by a uniport with compensating H^+ and K^+ efflux. Acidic amino acids are transported with 2 H^+ and the efflux of 1 K^+ (Wyse and Komor, 1984). In Avena sativa the transient depolarizations resulting from amino acid supply (1-2 minute duration) are followed by repolarizations which are complete within 2-3 minutes (Kinraide et al., 1984). These authors suggest that the repolarizations are a result of efflux of K^+ in a passive manner.

7.1.3. Transport of nitrogen into the vacuole

A large body of evidence suggests that the vacuole is an important site for the storage of at least nitrate. Studies on the uptake of NO_3^- have been interpreted to suggest that the control of this process originates from the regulation of transport across the tonoplast (Glass et al., 1985). These authors report that NH_4^+ strongly inhibited the uptake of NO_3^- and that NO_3^- influx across the plasmalemma of barley roots was insensitive to tissue NO_3^- and Cl^- status; inhibition of tonoplast uptake led to higher cytoplasmic contents and thus greater translocation to the shoot.

Plasmalemma and tonoplast H^+ - ATPases involved in the production of electrogenic gradients have been established. These activities may represent electrogenic proton pumps which have been postulated to generate proton electrochemical gradients which may drive the transport of ions and small molecules (Lew and Spanswick, 1984). The plasmalemma form is sensitive to vanadate, molybdate and azide and insensitive to NO_3^- while the tonoplast ATPase H^+ pumping is stimulated by Cl^- and inhibited by NO_3^- (Zocchi, 1985). No explanation of the effect of Cl^- has been forthcoming, but the effect of NO_3^- has been found to be competi-

tive in nature (Griffith et al., 1986). Nitrate, because of its trigonal planar geometry and thus its similarity to the terminal phosphate of ATP, has been suggested to competitively occupy the active site of ATPase. Conditions producing protein phosphorylation (Ca^{2+} with calmodulin and a protein kinase) have been reported to inhibit total and ionophore stimulated ATPase activity (Zocchi, 1985). Activity of the NO_3^- sensitive form of the ATPase was found to be more depressed than the activity of the vanadate form suggesting that the tonoplast ATPase is more sensitive than the plasmalemma form to phosphorylation. The tonoplast form is unaffected by the supply of AMP (Raush et al., 1985 and Zocchi, 1985) but ADP is a potent competitive inhibitor (K_i 0.18 mM) (Raush et al., 1985). The proton pumping ability of this ATPase is inhibited by nitrate, but this inhibition does not prevent the electrophoresis of this ion into the vacuole (Lew and Spanswick, 1985). Nitrate uptake has been shown by these authors to be a saturable phenomenon and relatively specific in that only certain anions (KCl and KNO_3) could induce the associated proton uptake (Lew and Spanswick, 1985).

Vacuoles, traditionally considered a storage and detoxification compartment, may function as a multifunctional compartment involved in many metabolic processes. The vacuole has a limited number of anabolic enzymes but has many hydrolytic enzymes and transport systems involved in vacuolar accumulation across the tonoplast (Boudet et al., 1984). Vacuolar storage allows intracellular accumulation of end products without the associated feedback effects and may serve for reversible and irreversible storage, the former of which may occur over long term (storage for periods of active growth) or short term (accumulation for temporarily separated utilization). It has been noted that NO_3^- is often stored by species from arid environments where the NO_3^- is accumulated after rain, possibly to maintain growth during the dry period (Smirnoff and Stewart, 1985).

In unicellular green algae without major storage vacuoles supply of ABA has been found to stimulate the uptake of NO_3^- by up to 200%. Although this stimulation was proposed to be the result of the activation of H^+ extrusion at the plasmalemma, no evidence

for this was found and the effect of A⁺ was more directly related to increased starch degradation to form glucose and thus respiratory activity (Ullrich and Kunz, 1984). ABA had very little effect on photosynthetic O₂ evolution, NO₂⁻ reduction and NH₄⁺ assimilation (Ullrich and Kunz, 1984).

Nitrate may accumulate in some circumstances in crop plants (spinach, beet, radish) to 10-24% dry weight (Martinoia et al., 1981 and Smirnov, 1985). 'Normal' levels of NO₃⁻ in plant tissues are in the range 0-140 μmol.g dry weight⁻¹ or 0-0.2% of dry weight and accounts for 0-10% of plant nitrogen (Smirnov and Stewart, 1985). High nitrogen fertilization can lead to accumulation of NO₃⁻ to such an extent that the plant tissue is toxic if consumed (Smirnov and Stewart, 1985). This accumulated NO₃⁻ does not mix with the existing pools of nitrate, but is found to be required for the maintenance of a fully induced NR. Vacuoles isolated from protoplasts have been shown to contain much (99%) of the cellular NO₃⁻ (Martinoia et al., 1981) and thus the 'storage' and 'metabolic' pools are probably separated by the tonoplast.

The *in vivo* assay for NR depends on the accumulation of NO₂⁻ under anaerobic conditions. With the supply of NO₃⁻ this activity continues for in excess of 1 hour, but in the absence of supplied NO₃⁻ the reaction quickly (<1 hour) runs down, although supply of NO₃⁻ or monohydroxy alcohols causes a resumption of activity. In addition the amount of NO₂⁻ accumulated is only a small fraction of the amount of NO₃⁻ present in the tissue (Ferrari et al., 1973). Gradual leakage of NO₃⁻ out of the tissue could not account for this because NO₂⁻ production continued even after the cessation of leakage. These results have been taken to suggest the presence of a small pool of NO₃⁻ available for reduction, while the rest of the tissue NO₃⁻ is in storage (Ferrari et al., 1973). The effects of the alcohols on NO₂⁻ accumulation were suggested to be the result of increased leakage of NO₃⁻ from storage to the metabolic pool (Ferrari et al., 1973). It has however been shown that different types of alcohols have different effects. Gray and Cresswell (1983) found that while both propanol and ethanol promoted NO₃⁻ utilization, propanol brought about an ac-

cumulation of NO_2^- under both aerobic and anaerobic conditions while ethanol caused an accumulation of NO_2^- only under anaerobic conditions in Z. mays. In contrast Yoneyama (1981) reported that propanol only increased NO_2^- accumulation under aerobic conditions and depressed NO_3^- utilization for several species. Even if the differential effects of these alcohols do exist, it is still uncertain what the effects of the alcohols on NO_3^- and NO_2^- metabolism are, except that they may be more complex than suggested by the leakage of NO_3^- into the metabolic compartment. It is possible to speculate that the differences may arise from the metabolism of ethanol under aerobic conditions. From experiments in which the plant cells were washed repeatedly it was concluded that the metabolic pool of NO_3^- is cytoplasmic while the storage component is probably vacuolar (Ferrari et al., 1973). Interpretation of these results is however open to question as it has been recently shown that even under anaerobic conditions, NO_2^- is metabolised, and thus the lack of NO_2^- accumulation is not a reliable indicator of the flux of nitrate (Yoneyama, 1981; Gray and Cresswell, 1983; Ben-Shalom et al., 1983). In addition to the trophic function of nitrate, this ion may play a rôle as a cellular osmoticum (Smirnov and Stewart, 1985).

The high NO_3^- content of some leaves means that it may be as important as other major anionic components of leaf osmotic potential (Smirnov and Stewart, 1985). Boudet et al. (1984) have shown an inverse relationship between NO_3^- and malate and have suggested that malate balances NO_3^- uptake into vacuolar storage. The relationship between NO_3^- and malate may exist for other reasons discussed later. A similar inverse relationship between sugar concentration and NO_3^- has been reported in Lolium multiflorum (Veen and Kleinendorst, 1985). Blom-Zandstra and Lampe (1985) found evidence suggesting that NO_3^- may serve as an osmoticum at low light intensity to compensate for a decline of carbohydrate concentration. Nitrate assimilation has also been suggested to contribute toward the avoidance of photoinhibition. A light dependent ATPase maintained pH gradient has been found between the cytoplasm and the vacuole and may explain many of the energetically uphill transport systems operating on the tonoplast and some of the control mechanisms (Boudet et al., 1984). Al-

though the NO_3^- readily permeates the vacuole with the expenditure of ATP, NO_3^- cannot passively diffuse out of this compartment.

7.2. NITROGEN EFFECTS ON CARBON METABOLISM THROUGH UPTAKE MECHANISMS

The apparent interdependence of NR and transport of NO_3^- makes it difficult to distinguish between the effects of NO_3^- on carbon metabolism and the effects on transport, because carbon may be involved in both functions. Nitrate reduction has been reported not to occur or not to be assimilated further than NH_4^+ under conditions of CO_2 deprivation. Nitrate derived NO_2^- and NH_4^+ have been reported to accumulate in the external medium of Chlorella (Di Martino Rigano et al., 1985) and Scenedesmus (Larsson et al., 1985a) suspensions kept under conditions of CO_2 deprivation. This effect has often been reported to be pH dependent with no accumulation occurring at pH 6.4. Accumulation of NO_3^- occurs at pH 8.2 but the products of reduction are returned to the medium as NH_4^+ (Di Martino Rigano et al., 1985). Supply of light and glucose to barley seedlings has been reported to induce NR. This induction occurred without exogenous NO_3^- when light was supplied, while glucose was only effective if NO_3^- was present (Aslam et al., 1976). These results were interpreted by the authors to imply that the metabolic pool was replenished from a storage pool in the presence of light while glucose and light are both effective in the transport of extracellular NO_3^- into the metabolic pool. Eisele and Ullrich (1977) reported a glucose reversible inhibition of NO_3^- uptake and reduction, especially at low pH. The effect of glucose was found not to be markedly pH dependent and high levels of CO_2 (1%) exhibited the same effect. Whether this effect was primarily the result of an effect on NO_3^- uptake or NO_3^- reduction is difficult to assess. It is unlikely however that the external pH has a strong effect on the reduction of NO_3^- and thus it would seem that the effect is localized in the plasmalemma (Eisele and Ullrich, 1977). In addition NR has a pH optimum of between 7 and 8 which is markedly different from the pH optimum shown for NO_3^- uptake (Eisele and Ullrich, 1977).

The further reduction of NO_2^- is likely to be even further buffered against external changes in pH because of its localization in the chloroplast. Thus the effect of glucose addition and elevated levels of CO_2 may exert their effect through an intermediate common to the pathways for the metabolism of both substances (Eisele and Ullrich, 1977). These authors propose that a carrier molecule responsible for NO_3^- uptake is stimulated by the intermediate. The production of an OH^- during the complete reduction of NO_3^- is constant with pH and thus the efflux of the OH^- and the uptake of NO_3^- either has to occur up a concentration gradient or against a membrane potential. Thus the uptake and reduction of NO_3^- must be an active process (Eisele and Ullrich, 1977).

In Chlorella the accumulation of nitrate, NO_2^- and NH_4^+ at pH 8.2 resulted in no excretion into the external medium and this led to the proposal that the effect of pH is on the mechanism of transport and not the availability of either carbon to supply reductant or as an organic acceptor molecule for the reduced nitrogen. It seems unlikely that carbon availability would be controlled by the extracellular pH (Di Martino Rigano et al., 1985). A permease, labile at low pH, has been proposed to explain these results. An alternative explanation is that a permease is normally controlled by the cellular carbon status, but at high pH this control is lost. Larsson et al. (1985b) reported that NO_3^- uptake and/or reduction was stimulated by CO_2 at non-limiting light intensities ($>100 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) associated with reduced NH_4^+ efflux. They also report a reduced uptake and reduction of NO_2^- and a positive correlation between NH_4^+ uptake/reduction with CO_2 concentration. These authors commented on the proposal that a C-N intermediate dependent on the ratio of carbon to NH_4^+ , plays a regulatory rôle in the cell and extended this proposal to include the effect of NO_3^- . The control of the uptake of NO_2^- is suggested to be unlikely as this species can apparently cross the chloroplast envelope freely as the undissociated anion (Larsson et al., 1985b). Kaiser and Heber (1983) reported sensitivity of photosynthetic O_2 evolution to both bicarbonate and nitrite. In examining the effects of NO_2^- on photosynthesis it soon becomes evident that pH considerations are very important. In studies of

the pH sensitivity of protoplast and chloroplast photosynthesis it was found that protoplasts were more tolerant of pH changes than chloroplasts, although both systems demonstrated a capacity for controlling the intracellular pH. The utilization of bicarbonate is strongly pH dependent due to the predominance of HCO_3^- at high pH. Evidence indicates that CO_2 is the species which is transported across the plasmalemma (Kaiser and Heber, 1983) and not HCO_3^- and thus lower pH favours uptake of carbon dioxide from the medium. High concentration of HCO_3^- (30 mM) have been found to lead to stromal acidification owing to the loss of OH^- through the stromal formation of HCO_3^- from CO_2 (Kaiser and Heber, 1983). Similarly NO_2^- entering the stroma would result in acidification. In this regard it is important to establish whether NO_2^- or HNO_2 enters the chloroplast. Kaiser and Heber (1983) concluded from theoretical calculations that both the protonated and the unprotonated forms can cross the plasmalemma and chloroplast envelope, although anions crossed 10^5 times more slowly. The plasmalemma was found to be less permeable to NO_2^- than the chloroplast by a factor of 200. It should be noted that the experiments of Kaiser and Heber (1983) were conducted at extremely high concentrations of NO_2^- (up to 100 mM).

Incubation of unicellular green algae with glucose or in the presence of light has been found to enhance the consumption of NO_3^- (Schlee et al., 1985) and this enhancement has been attributed, in most cases, to the provision of energy or carbon skeletons for NO_3^- reduction. Glucose has recently been reported however to induce general amino acid transport and NH_4^+ transport and a similar mechanism has been proposed for NO_3^- accumulation (Schlee et al., 1985). The effect of glucose on NO_3^- uptake (5-fold increase) was only apparent after a 40 minute incubation period although glucose did not influence the uptake rate. Thus the effect of glucose appears to be only in induction. The uptake of NO_3^- was accompanied by the operation of an H^+ symport in a stoichiometry of 1:1 but no K^+ movement was observed (Schlee et al., 1985). Thus the uptake of NO_3^- may be an electroneutral process.

7.3. ACCUMULATION OF IONS ASSOCIATED WITH INORGANIC NITROGEN REDUCTION

Considerable attention has been given to the accumulation of various ionic species during the reduction of various nitrogen species. The accumulation of these ionic species has been accorded much importance by some authors (eg. Raven and Smith, 1976; Smith and Raven, 1979; Smirnoff and Stewart, 1985). Although the production of these ions undoubtedly occurs during nitrogen reduction, it is hard to conceive that these ions should present a major problem to the plant considering the vast number of redox and acid/base reaction which occur. In spite of these reservations, the literature dealing with this subject is briefly reviewed below.

Nitrate assimilation generates OH^- ions (1 per NO_3^-) and NH_4^+ assimilation generates H^+ (1 per NH_4^+) which must be excreted or neutralized to maintain a constant cytoplasmic pH (Raven and Smith, 1976; Smith and Raven, 1979). Although the cell walls could store some of the H^+ and OH^- , this could only account for a fraction of that produced. For the roots this problem is simply overcome through the excretion of bicarbonate or hydroxyl into the bathing medium (Smirnoff and Stewart, 1985) and this excretion may even be linked to the uptake of anions such as NO_3^- (Ben-Zioni et al., 1971).

The distribution of nitrogen reductive sites has been proposed to be the result of selective pressures related to the requirement of the plant for the disposal of excess H^+ and OH^- (Raven and Smith, 1976). The site of NH_4^+ assimilation is predominantly the root and N is transported to the shoot as a mixture of amino acids and their amides. Ammonium assimilation by the root involves the acidification of the rooting medium (Raven and Smith, 1976). If NH_4^+ assimilation is to occur in the shoot sucrose translocated from the shoot may be converted into malate in the root releasing 2 H^+ which may be exchanged for NH_4^+ ions and the malate is translocated to the shoot where it enters the pH stat and is decarboxylated to yield pyruvate. This pH control may be mediated through the pH effects on PEPc and malic enzyme (Smith

and Raven, 1979). Raven and Smith (1976) concluded that all the H^+ produced in the reduction of NH_4^+ could not be stored in the vacuole for osmotic and pH reasons and thus the predominant means of detoxification appears to be through root excretion.

Reduction of NO_3^- actually results in the formation of $2 OH^-$, but the production of H^+ by the subsequent assimilation of NH_4^+ results in a net stoichiometry of only $1 OH^-$ per NO_3^- . This assumes that NO_3^- reduction and NH_4^+ assimilation occurs in the same pH pool, which is probably not the case. Although NO_3^- assimilation can occur in the roots, depending on external NO_3^- concentration, it appears that the major site of NO_3^- reduction is the shoot for energetic reasons. It is presumed inefficient to transport sugars to the root and then have to transport the products of NO_3^- assimilation to the shoot and it is assumed that light intensities are generally sufficient to saturate photosynthesis for provision of reductant to NR in the leaf (Smirnoff and Stewart, 1985). Whether translocation of NO_3^- occurs or not is dependent on the external NO_3^- concentration and the root temperature as discussed previously. The reduction of NO_3^- in the root provides none of the problems associated with the leaf in terms of the disposal of OH^- . The OH^- produced in the root is either excreted to the external medium or metabolised via the biochemical pH stat. The assimilation of NO_3^- in the root is proposed to lead to a drop in pH which activates PEPc which neutralizes OH^- with the production of malic acid. In the shoot efflux accounts for 50% in maize and 10% in *Beta vulgaris* of the generated OH^- , and the balance is thought to be accounted for by biochemical pH stat.

With increasing NO_3^- assimilation, the bulk of the anions (OH^-) produced accumulated in the plant as malate with a parallel accumulation of cations, while efflux of OH^- accounted for only 20% of the anion charge (Kirkby and Knight, 1977). The stimulation of cation uptake was proposed to result from a requirement for an accompanying ion for shoot translocation. In addition, the cation was suggested to balance the production of organic acids formed during NO_3^- reduction (Kirkby and Knight, 1977). The implication of this proposal is somewhat different from that of Ben-Zioni et

al. (1971) where the accompanying cation (K^+) was suggested to be retranslocated downward in the phloem. Here the cations are suggested to accumulate in the shoot.

In the leaf organic acids may be stored in the vacuoles as salts or be translocated to the phloem and on down to the roots where they may be decarboxylated and the resulting hydroxyl excreted, possibly in exchange for further NO_3^- (Ben-Zioni et al., 1971). These authors propose that the stoichiometric production of malate with NO_3^- reduction allows the movement of K-malate to the root where the malate is oxidised to yield $KHCO_3$ which may then exchange for KNO_3 . NO_3^- feeding has been reported to result in an increase of K^+ and inorganic acid content (aconitate and malate) of maize leaves (Morot-Gaudry et al., 1985). Criticisms leveled against this suggestion include the observation that significant root NO_3^- reduction occurs and tracer studies in tomatoes did not show the cycling of organic acids (Kirby and Knight, 1977; van Beusichem et al., 1985). In Ricinus communis it was shown that the efflux of HCO_3^- was strongly dependent on the supply of NO_3^- and that K^+ recirculation was significant (Kirby and Armstrong, 1980). On the other hand, excretion from the same species was found to only account for 47% of the anion charge and significant root reduction of NO_3^- was observed (van Beusichem et al., 1985). The same authors found that only 24% of excreted HCO_3^- could be attributed to shoot borne organic anions. Thus different species appear to support different mechanisms and the interpretation of results is, in some cases, somewhat dubious.

It has been suggested that the OH^- excretion from the root is the result of NO_3^- reduction in this organ alone (Raven and Smith, 1976). The formation of calcium oxalate from malate allows the deposition (precipitation) of this salt in the vacuole thus avoiding the associated osmotic potential problems. The formation of OAA is catalysed by the enzyme PEPc which is found in the cytoplasm and in some cases the chloroplasts of C_3 and C_4 mesophyll cells which are in the latter case also the site of NO_3^- and NO_2^- reductase (Smirnoff and Stewart, 1985). The absence of PEPc from the bundle sheath, and thus the lack of a pH stat, has been suggested to account for the lack of NO_3^- reduction in

these cells. This compartmentation has been further suggested to spatially separate NO_3^- reduction and carbon assimilation by RuBPC preventing competition between the two processes (Smirnov and Stewart, 1985). These considerations obviously do not apply to C_3 plants. There are however many other possibilities through which competition may be avoided. C_4 plants do not exhibit saturation of photosynthesis even at high light intensities. Thus there may be insufficient photochemical energy to allow competition between nitrogen and carbon assimilation. Mechanisms to avoid such competition in C_3 plants have been suggested to include the following:

- a) Nitrate assimilation most active when light intensity maximal and CO_2 fixation at a minimum (e.g. at noon in some plants)
- b) Nitrate assimilation has been suggested (perhaps naively) to dissipate excess photochemical energy in place of photorespiration
- c) Nitrate enhancement of the photosynthetic light reactions may provide enough reductant to avoid competition between nitrogen and carbon assimilation (Smirnov and Stewart, 1985).

The use of a biochemical pH stat involves the partitioning of large quantities of carbon into storage in the vacuole. Oxalate is the most efficient because it can neutralize one OH^- for each carbon. Up to 10% of the organic carbon may be involved in organic acid formation. The sequestering of this organic acid in the vacuole has been estimated to consume about 5-10 ATP/N while transport of carbon to root probably only requires 2 ATP (Smith and Raven, 1979). Simultaneous supply of NO_3^- and NH_4^+ would alleviate the pH stress imposed by supply of one of the two forms. This may in part account for the synergistic effects of NO_3^- and NH_4^+ supply on growth.

7.4. EFFECTS OF INORGANIC NITROGEN ON METABOLISM OF OTHER IONS

Inorganic phosphate is known to be essential for the translocation of photosynthates out of the chloroplast. Reduced supply of phosphate has been shown by several workers to be related to the accumulation of starch in the chloroplast (Heldt et al., 1977; Ariovich and Cresswell, 1983). Thus anything affecting the availability of P_i in the cell is also likely to have wide ranging effects on carbon metabolism. NO_3^- has been reported to inhibit the accumulation of P_i in corn roots. This was suggested to be the result of reduced exchanges between the symplasm and vacuoles where NO_3^- inhibited the transport of P_i from the cytoplasm to the vacuole leading to an increase in cytosolic phosphate (Lamaze et al., 1984). This may have significance for the rôle of NO_3^- in regulating the efflux of photosynthate from the chloroplast. Nitrate has been proposed to utilize triose-P produced in the chloroplast as a source of reductant (Klepper et al., 1971) so that NO_3^- is expected to have an independent rôle. Phosphate is known to be required for the functioning of the DHAP/PGA shunt across the chloroplast membrane and has been suggested to be a major controlling influence on the levels of chloroplastic triose-P (Flügge and Heldt, 1977). CO_2 dependent O_2 evolution is strongly dependent on the adequate supply of P_i and passive leakage of P_i from the chloroplast drastically reduces this O_2 evolution (Mouriaux and Douce, 1981). Furthermore, the rates of photosynthetic carbon assimilation have been shown to be strongly influenced by the lack of either NO_3^- or phosphate and this has been related to the accumulation of starch under these conditions (Ariovich and Cresswell, 1983). Thus a further link between triose-P transport and NO_3^- may exist through the effects of this anion on phosphate accumulation in the cytosol.

7.5. THE EFFECTS OF NITROGEN ON ENZYME ACTIVITIES ASSOCIATED WITH CARBON DIOXIDE ASSIMILATION

The effects of inorganic nitrogen nutrition or short term supply on photosynthesis and photorespiration are not only due to the direct participation of nitrogen in biochemical reactions, but have also been attributed to the influence of nitrogen on enzymes associated with these processes. Active transport of HCO_3^- which is sodium inducible has been described in Anabaena cells (Reinhold et al., 1984). Although it is possible carbonic anhydrase plays an important rôle in aquatic plants (Salvucci, 1983), it appears that the major permeant carbon dioxide species in Asparagus mesophyll cells is CO_2 with HCO_3^- contributing to only 5-16% of the total net inorganic carbon uptake at high pH (8.5) (Espie et al., 1986; Espie and Colman, 1986). Similar results have been reported for Chlamydomonas reinhardtii (Marcus et al., 1984). Thus the possibility of direct effects of inorganic nitrogen on an HCO_3^- uptake system for higher plants can be neglected.

A close relationship between photosynthetic rate, quantity and activity of RuBPC exists (Avdeeva and Andreeva, 1973). Vaklinova et al., (1981) report that NO_3^- and NH_4^+ ions enhance photosynthetic and photorespiratory activity in pea protoplasts and barley leaves. This was found to be correlated with increases in RuBPC/o activities. Supplied nitrogen of unspecified form has been reported to activate PEPc, RuBPC and glyceraldehyde phosphate dehydrogenase (expressed per gram fresh weight) in both C_3 (broad bean) and C_4 (Zea mays) plants (Avdeeva and Andreeva, 1973; Sugiyama et al., 1984). Fair et al (1974) reported that increasing NO_3^- and NH_4^+ concentration in the medium of Hordeum vulgare resulted in higher activity of RuBPC, glycolate oxidase and catalase (expressed per gram fresh weight) while RuBPC activity was reported to increase in H. vulgare (Tew et al., 1974). Similarly PEPc, glycolate oxidase and RuBPC activities (expressed per gram fresh weight) have been reported to increase with both NO_3^- and NH_4^+ supply to Zea mays, Themeda triandra, Hyparrhenia hirta and Eragrostis curvula (Cresswell et al., 1977). The same authors report a change in the ratio of carboxylase to oxygenase

activities of RuBPC/o in all the species studied and with both NO_3^- and NH_4^+ . This was suggested to be the result of an allosteric change in the enzyme as no significant changes occurred in the activity of RuBPC. This increase in activity of RuBPC was associated with an increase in carbon dioxide compensation points and increased sensitivity to oxygen inhibition of net photosynthesis. Shieh and Liao (1985) reported little effect on CO_2 assimilation in rice on the supply of NH_4NO_3 over a wide range of concentrations although growth, RuBPC activity, PEPc activity, NR and NiR activities were found to increase. In spite of the increase of RuBPC activity, specific activity of this enzyme and that of RuBPC remained fairly constant.

Apart from the rôle of PEPc in carbon assimilation, it is largely assumed that this enzyme, with malate dehydrogenase and malic enzyme, functions as a cellular pH stat in C_3 plants (Schweizer and Erismann, 1985). PEPc is also postulated to function as an anaplerotic enzyme for the TCA cycle from which carbon may be derived in some tissues for NH_4^+ assimilation (Schweizer and Erismann, 1985). The extractable activity of PEPc was enhanced in leaf tissue of NO_3^- fed plants but not in NH_4^+ fed plants. Roots of NO_3^- fed plants showed no increase in PEPc activity while NH_4^+ fed plant root PEPc responded positively (Schweizer and Erismann, 1985). These results may be interpreted as meaning that NH_4^+ requires a pH stat or carbon from the TCA in the roots while no such requirements are made in the leaf because NH_4^+ is assimilated into amino acids in the roots. Nitrate on the other hand is assimilated in the shoots of some plants and may therefore require a pH stat in this tissue. Schweizer and Erismann (1985) reported no effect of inorganic nitrogen on RuBPC.

No consideration of the mechanism of these effects exists in the literature and a general elevation in protein synthesis on supply of various nitrogen forms cannot be excluded. The reduction of NO_3^- produces OH^- in cells whereas assimilation of NH_4^+ produces H^+ . The chloroplastic system is remarkably sensitive to pH changes and such a release of cations/anions could have important consequences (Schweizer and Erismann, 1985). PEPc is largely assumed to function as a pH stat in C_3 plants and is also at-

tributed an important anaplerotic rôle in the TCA cycle. Schweizer and Erismann (1985) found differential effects of NO_3^- and NH_4^+ on PEPc and pyruvate kinase (expressed on the basis of both fresh mass and soluble protein) which they suggest may be related to a requirement for a pH stat in cells supplied with NO_3^- and NH_4^+ . No effects of NO_3^- and NH_4^+ on RuBPC were found. Differential effects of NO_3^- and NH_4^+ on the carboxylation enzymes and the alteration in the ratios of RuBPC/RuBPo may point to other mechanisms than just an elevation of general protein synthesis on supply of nitrogen nutrition.

7.5. DIRECT EFFECTS OF INORGANIC NITROGEN ON THE LIGHT REACTION OF PHOTOSYNTHESIS

Direct effects on the light reaction of inorganic nitrogen, particularly in the the form of NO_3^- , are not often considered. It has been known for some time that NO_3^- stimulates oxygen evolution (Warburg and Negelin, 1920 in Osman et. al., 1982), but it is an open question as to whether this stimulation is exclusively due to an acceleration of NO_3^- reduction and the concomittant demands on the light reaction for reducing potential or more direct effects. It has been suggested that NO_3^- could act as an electron acceptor for the Hill reaction or replace the Cl^- anion in the water oxidation system (Osman et al., 1982).

Addition of NO_3^- to a suspension of salt depleted thylakoids incapable of NO_3^- reduction raised the rate of O_2 evolution by a factor of 2-3. Through the use of inhibitors and artificial electron donors/acceptors it was determined that the site of NO_3^- action was at the water oxidation step or the donor side of PSII (Osman et al., 1982). In these studies NO_3^- was supplied at 10 mM while other authors have applied much higher concentrations (100 mM) and obtained inhibitory responses (Stemler and Murphy, 1985).

High concentrations of various anions inhibit PSII electron transport. Nitrate supplied as KNO_3 inhibited O_2 evolution in a competitive bicarbonate reversible fashion. This was attributed to the binding of NO_3^- to site(s) on PSII (Stemler and Murphy,

1985) probably located between QA and the PQ pool (Van Rensen and Snel, 1985). Supply of HCO_3^- has been shown to stimulate Hill reaction activity (Shin et al., 1985). Removal of CO_2 from the culture medium of *Chlamydomonas stellata* resulted in an initial diminishment of photosynthetic electron transport at the oxidising side of photosystem II followed by an additional effect on the reducing side of the photosystem (Mende and Wiessner, 1985). The effects of HCO_3^- on PSII have been related to the provision of H^+ to the reaction site where HCO_3^- functions as a shuttle between the aqueous phase and QB or alternatively HCO_3^- may cause conformational changes in the proteins involved with PSII (Van Rensen and Snel, 1985). A comparison between the effects of NO_3^- at high and low concentrations and the different modes of action between the bicarbonate reversible and water oxidation step shows that NO_3^- may exhibit specific effects at this level.

Low concentrations of NH_4^+ are known to uncouple photophosphorylation and higher concentrations inhibit the water oxidation step of the light reaction (Izawa, 1977). The uncoupling effect is a purely physical process resulting from the protonation of the NH_3 within the thylakoid system which results in the depletion of the proton gradient while inhibitory effects are related to the effects of NH_3 on the manganese center responsible for O_2 evolution (Izawa, 1977). The uncoupling effect applies to other systems (eg. cyanobacteria) with a small closed space in which H^+ gradients are important (Barskii et al., 1985).

THE INTERDEPENDENCE OF INORGANIC NITROGEN REDUCTION AND CARBON METABOLISM

7.7. REDUCTION OF NITRATE

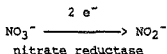
7.7.1. Light energy dependent nitrate reduction

The rôle of the chloroplast in the reductive assimilation of CO_2 is well known. This process is dependent on the provision of ATP and reducing equivalents (NAD(P)H) by the light reaction. The

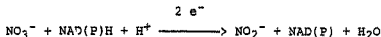
light reaction occurs in the thylakoid membrane system and releases ATP, NAD(P)H and $Fd_{(rd)}$ into the stroma of the chloroplast (Anderson, 1981). There the high energy phosphate and reducing equivalents are available for the reductive assimilation of CO_2 as well as a variety of other processes. Quantitatively one of the most important reductive processes is the assimilation of NO_3^- to NH_4^+ (Anderson, 1981). The initial reduction of NO_3^- to NO_2^- probably occurs in the cytoplasm with NAD(P)H as the electron donor. The reducing power for this reduction may be supplied from the chloroplast or from the respiratory processes (Anderson, 1981). Because of the dependence of the cytoplasmic compartment on the chloroplast for the provision of respiratory sub it becomes very difficult, but possible, to distinguish in the two compartments.

7.7.2. Reduction requirements of Nitrate Reductase

The reduction of NO_3^- to NO_2^- is facilitated by the metaloprotein NR in a 2 e^- step.

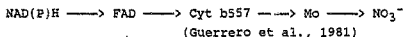


The NR found in higher plants is pyridine nucleotide dependent and the enzyme is soluble (Guerrero et al., 1981). The enzyme has a diaphorase activity resulting in the reduction of a variety of electron acceptors by NAD(P)H and a NR ability which is pyridine nucleotide independent resulting in reduction of NO_3^- by reduced flavins or viologens (Guerrero et al., 1981). Both moieties participate jointly and sequentially in the transfer of electrons from NAD(P)H to NO_3^- (Guerrero et al., 1981). The reaction mechanism is ping-pong (bi-bi) so NADH binds before NO_3^- (Hewitt et al., 1979). Once NADH is bound to the enzyme and NO_3^- also attached, the second site of the enzyme for NAD(P)H becomes unavailable in the presence of NO_3^- .



$$\Delta G_0, \text{pH } 7 = -34 \text{ kcal.mol}^{-1}$$

FAD, cytochrome b557 and molybdenum have been identified as essential components of NR (Hewitt, 1975). The FAD component is associated with the diaphorase moiety while the Mo is thought to be the site at which NO_3^- binds. $\text{Mo(VI)}/\text{Mo(IV)}$ is probably the proximal reductant of NO_3^- . The cytochrome b557 component is reduced by NAD(P)H and oxidised by NO_3^- and has thus been proposed to mediate electron transfer between FAD and NO_3^- .



Nitrate reductase capable of utilizing NADH occurs in both root and shoot material although the enzyme from different tissues exhibits subtle differences (Oaks, 1979). This author reported that root NR was localized in the cytoplasm in the soluble phase. Although ATP is not obviously involved with the reduction of NO_3^- , ADP and Pi have been reported to play a rôle in the regulation of NR activity. ADP inhibited NR where AMP and ATP were relatively ineffective (Chaparro et al., 1976). The inhibition of NR activity by ADP is reversibly determined by the concentration of NADH and thiols in the system (Baglesham and Hewitt, 1971). The effects of ADP are complex and inhibition is both competitive with NADH and non-competitive for NADH plus NO_3^- (Hewitt et al., 1979). Two separate sites on the enzyme react with ADP and the competitive effect is co-operative while the noncompetitive effect is linear and abolished by thiols. The inhibitory capacity of ADP is however low (15-18%) in the dark and nonexistent in the light due to its concentration. Both glutamine and fructose diphosphate play some uncertain rôle in modifying this effect (Hewitt et al., 1979). Pi on the other hand was shown to increase reductase activity under high reducing potential conditions. ADP was proposed to play a rôle in allosteric regulation of NR to facilitate a balance between NO_3^- reduction and oxidative phosphorylation (Nelson and Ilan, 1969). Sawhney et al., (1978)

proposed that the levels of ATP regulate the supply of NADH for NO_3^- reduction through feedback on mitochondrial respiration allowing greater partitioning of NADH into NR than oxidative phosphorylation. An alternative possibility is that low ATP restricts the permeability or attachment of a proposed membrane bound compartment containing NR to the chloroplast thus causing a cytoplasmic accumulation of NO_2^- (Soares et al., 1985a). Dry et al. (1981) reported no direct requirement of ATP for NO_2^- reduction but did find a requirement for ATP regulated G6P.

The type of pyridine nucleotide required for NO_3^- reduction has been the subject of much controversy. This question has added importance because of the production of NADPH by the chloroplast and NADH and NADPH in the cytoplasm from glycolysis and the pentose phosphate shunt respectively. In most higher plants NO_3^- reduction is catalysed by NADH:NR which is specific for NADH and has a relatively low K_m for NO_3^- (Redinbaugh and Campbell, 1981). The simultaneous presence of an NADH specific and a bispecific NAD(P)H:NR with a higher K_m for NO_3^- has been reported. The evidence for a specific NADPH:NR is contradictory (Redinbaugh and Campbell, 1981). Some of the NAD(P)H forms reported may be NADH specific forms where NADPH is converted to NADH by phosphatases during the assay (Dailey et al., 1982). In barley when lactate dehydrogenase and pyruvate were added to mop up NADH, NADPH activity of NAD(P)H:NR was reduced by 80-95%. There is however a small capacity for using NADPH by barley which could not be explained through the mediation of phosphatases (Dailey et al., 1982). Over reduction of the NR enzyme by NAD(P)H is inhibitory. Various other inhibitory effects on the enzyme become apparent in the presence of NAD(P)H and NO_3^- or various thiols (Eaglesham and Hewitt, 1971).

The rôle of the two types of NR has recently received more attention and the requirements for specific pyridine nucleotides linked to the occurrence of the enzyme. Apart from the inducible NR it appears that a constitutive component also exists (Hewitt et al., 1979) which does not require the presence of NO_3^- for its activity and is more tolerant of tungstate inhibition than the inducible form. Immunochemical characterization of NR from a

soybean mutant lacking the constitutive component has produced evidence that the constitutive component is NAD(P)H:NR and the inducible component NADH:NR (Robin et al., 1985).

7.7.2.1. Regulation of Nitrate Reductase

The activity of NR is regulated by a number of chemical compounds. Diverse mechanisms of control have been postulated. An inhibitor specific for NADH:NR, suggested to facilitate dark-light changes in NR activity, has been described for soybeans (Jolly and Tolbert, 1978). The inhibitor was non-competitive with NO_3^- , although it was associated with the enzyme, and became inactive in the light. An elegant and complex scheme for the control on NR involving the feedback from NiR involving cyanide and hydroxylamine has been suggested. Hydroxylamine was assumed to be formed from NO_2^- during NO_2^- reduction and the hydroxylamine was postulated to form cyanide in the presence of glyoxylate (Hewitt et al., 1979). The cyanide complexes 1:1 with NR resulting in complete inactivation. Cyanide inhibition may be removed by ferricyanide and rhodanese, which can transfer sulphur from thiosulphate to cyanide (Tomati et al., 1976). The possible physiological action of rhodanese may be through sulphation of cyanide to thiocyanate thus protecting NR. One of the features of the NR enzyme is that it can exist in two stable forms, one active and the other inactive. The interconversion between these two states appears to be governed by the levels of reductant and ADP (Chaparro et al., 1976). Ammonia, which functions as an uncoupler, results in the inhibition of NR activity and also causes an elevation of NAD(P)H levels in the cell (Chaparro et al., 1976) by 40 % in Chlorella (Hewitt et al., 1979). The uncoupling results both in an increase in NAD(P)H and a decrease in ATP/ADP ratios. This effect has been reported to be pH dependent (Radin, 1973) although this pH effect could be the result of the higher uptake of NH_4^+ at more alkaline pH (Mengel et al., 1983) and thus a greater effect on NAD(P)H levels. Reactivation is facilitated by white or blue light and it has been proposed that light aids the reoxidation of NR by exciting FAD which can then donate its electrons to O_2 (Aparicio and Maldonado, 1977). A direct effect of NH_4^+ on NR

has been suggested to occur, particularly in roots. It was not clear as to whether this effect was the result of the action of ammonium itself or whether the products of NH_4^+ metabolism were responsible because amino acids exhibited similar effects in some cases (Hewitt et al., 1979).

Nitrate has long been known to cause substrate induction of NR. It has been reported however that NO_2^- may activate NR and this activation has been proposed to occur at the molybdenum subunit while NO_3^- induces the dehydrogenase component (Kaplan et al., 1984). Induction of NR by NO_3^- is a protein dependent response (Hewitt et al., 1979). Mengel et al (1983) proposed that NR activity or synthesis is stimulated by the production of OH^- during NO_3^- reduction or through the production of organic anions. This was supported by the effects of pH and various anions on NR activity.

Glucose, sucrose and various other sugars have been reported to be effective in inducing NR. Glucose has been reported to be effective in inducing NR in maize roots (Puranik and Srivastava, 1983). The rate of NO_3^- uptake by Chlorella vulgaris was stimulated 5-fold by application of glucose but this was attributed to induction of an uptake system (Schlee et al., 1985). A specific increase in levels of NR by sucrose in bean leaves has been linked to direct induction of NR synthesis although it is also possible that sucrose may accelerate uptake of NO_3^- or mobilize the NO_3^- from a storage pool (Puranik and Srivastava, 1983).

A direct oxygen effect has been proposed which can be reversed or over-ridden by light (Reed and Hageman, 1977 in Hewitt et al., 1979). The rationale behind this postulate was the observation of the stimulation of the activity of the enzyme under dark anaerobic conditions. This type of oxygen repression is commonly observed in bacteria.

7.7.2.2. Alternative systems of nitrate reduction

An alternative scheme for the reduction of NO_3^- has been sug-

gested by Ivanova and Drobysheva (1985). The formation of the O_2^- anion radical occurs during biological oxidation. It is possible that up to 5% of the oxygen used during respiration can be reduced through the formation of superoxides (Ivanova and Drobysheva, 1985). In addition superoxides may be generated from the photolysis of water in the light reaction and other enzymatic systems and self-oxidising substances. The plant is generally protected from the effects of the superoxide through the detoxification effects of superoxide dismutase which catalyses the dismutation of superoxide to O_2 and H_2O_2 (Ivanova and Drobysheva, 1985). Nitrite accumulation in salt stressed plants was shown to be inversely related to superoxide dismutase activity (Kayupova et al., 1983). It has been suggested by Ivanova and Drobysheva (1982) that the superoxide may function in the reduction of NO_3^- to NO_2^- in situations where the protective detoxification performed by superoxide dismutase is not fully operational. Evidence for the possibility of such a pathway was derived from the observation that superoxides produced from peroxidase or xanthine oxidase systems could reduce NO_3^- . The system was however inoperative without the addition of diethyldithiocarbamate. Diethyldithiocarbamate becomes a free radical in the presence of superoxide and has been proposed to reduce NO_3^- . In previous work the same authors suggested that the O_2^- anion may itself reduce NO_3^- (Ivanova and Drobysheva, 1982). It is open to question as to whether the system is operational unless a thiol compound with similar properties to diethyldithiocarbamate functions in vivo and to what extent it may function.

7.7.3. The source of reductant for Nitrate Reductase

The source of reductant is of considerable importance because of the implications it may have for carbon assimilation and thus productivity. The source appears to depend on the type of tissue considered (i.e. leaf or root) and the presence and intensity of light. In addition any factor which alters the production or consumption of reducing potential will be of consequence. The literature on this subject is fraught with contradictions, some

of which at least have arisen from the experimental procedure for the assay of NR. The in vivo assay for NR measures the accumulation of NO_2^- under conditions (dark anaerobic) which supposedly prevent the reduction of NO_2^- . The NO_2^- accumulated is then taken as an indication of the amount of NO_3^- reduced, and thus the activity of NR. The assumptions made in this assay are :

- dark anaerobic conditions are not usual physiological conditions
- the assay assumes that NO_2^- is the only product of the reduction of NO_3^-
- NR activity is assumed to be unaffected by the conditions of the assay
- NO_2^- is assumed not to be metabolized under the conditions of the assay
- NO_3^- and NO_2^- are assumed not to leak from the tissue during the assay.

Questions may be raised as to validity of all of these assumptions. Ben-Shalom et al. (1983) and Gray and Cresswell (1983) have reported for instance that NO_2^- is further reduced under dark anaerobic conditions and in the presence of various inhibitors. Nitrate and NO_2^- have been reported to leak at significant rates from the tissue into the surrounding medium (Aslam, 1981). These authors also reported that the storage component of NO_3^- , not usually available for metabolism, may be made available through the effects of ethanol formed during anaerobic conditions. Evidence for this came from the stimulatory effects of propanol, ethanol and 2,4-dinitrophenol on NO_3^- reduction. Improved methods for NO_3^- determination are now available (Cataldo et al., 1975) and these may be used for an effective in vivo assay by measuring NO_3^- loss from the tissue (Soares et al., 1985a).

The two major tissues used (leaf and root) are very different with respect to their pathways for provision of reductant to NR principally as a result of the lack of the light reaction and carbon assimilation in the root. This review is principally concerned with events in the leaf but results obtained from roots cannot be ignored as they provide a valuable insight into the

control of NO_3^- reduction. In leaves the principal question is whether NO_3^- reduction is directly or indirectly dependent on the light and/or dark reaction of photosynthetic carbon assimilation.

The chloroplast envelope is impermeable to NAD(P)H (Walker, 1976) and thus direct export of light reaction produced reducing equivalents is not possible. Evidence for this comes from the ability of ruptured chloroplasts in contrast to the inability of intact chloroplasts to utilize NADP as a Hill oxidant (Walker, 1976). Similarly, rapid direct ATP translocation across the membrane does not occur. A slow and specific counter exchange involving ATP movement into the chloroplast probably for dark provision of ATP to the chloroplast does occur (Heber and Heldt, 1981). The site at which the blockage of movement across the chloroplast envelope occurs is probably the inner membrane. The outer membrane is reportedly permeable to metabolites of low molecular weight while the inner membrane provides a site of specific metabolite transport (Heldt and Sauer, 1971).

The impermeability of the chloroplast to these important high energy substances has led to the postulation of shuttles which would involve the movement of intermediates across the membrane. Light generated reducing equivalents and high energy phosphate are transported across the chloroplast envelope as reduced and phosphorylated metabolites (e.g., malate and DHAP), that are freely permeable to the chloroplast (Anderson, 1981). The occurrence of shuttles capable of transporting these metabolites in and out of the chloroplast is dependent on the presence of translocators in the chloroplast envelope. Two major systems are thought to be involved:

- a) a phosphate and phosphate ester translocator
- b) a dicarboxylate translocator (Heber and Heldt, 1981).

The phosphate translocator facilitates the transport of fixed carbon in the form of triose-phosphate or 3-phosphoglycerate from the chloroplast in exchange for inorganic phosphate or other phosphate ester (Heldt and Rapley, 1970). The phosphate esters transported are usually 3 carbon compounds (Heldt, 1976). These substances are transported as divalent ions and as a consequence

the transport is strongly pH dependent. The translocator appears to be a protein of 29 kD (Flügge and Heldt, 1977; 1978) with one inhibitor binding site and a turnover time of 5000 min^{-1} (Heber and Heldt, 1981).

A number of dicarboxylates such as L-malate, oxaloacetate, α -ketoglutarate, L-aspartate and L-glutamate are rapidly taken up by the chloroplast (Heber and Heldt, 1981). There is competition between these compounds for transportation indicating that the same carrier is involved (Heldt and Rapley, 1970). However incomplete inhibition of uptake of some, but not other dicarboxylates, points to a number of different carriers with overlapping specificity (Heber and Heldt, 1981). In addition to these two major translocators there also appears to be a specific glucose translocator functioning in the translocation of D-glucose (but not L-glucose) produced from starch degradation out of the chloroplast. This glucose translocator was strongly inhibited by high cytoplasmic glucose concentrations (Heber and Heldt, 1981). A pyruvate translocator has also been reported which functions in C_4 plants but not apparently in C_3 plants (Heber and Heldt, 1981).

Apart from the existence of these specific carriers and passive diffusion across the envelope, there exists the possibility that exocytosis and endocytosis may occur providing a relatively non-specific port of exit/entry. Various types of exosomes have been reported and the type and content depend on mineral nutrient supply and light intensity (Selga, 1983). Entry of substances from the apoplast by endocytosis was also observed by the same authors.

7.7.3.1. The C_4 dicarboxylic acid (malate/oxaloacetate) shuttle

Leaf tissue has the ability to reduce OAA to malate in the light and this process has been shown to be linked to the photoproduction of O_2 by the light reaction (Anderson and Done, 1978). Reduction of OAA is dependent on light coupled NADP-MDH as evidenced by the promotion of OAA-dependent O_2 evolution by NADPH

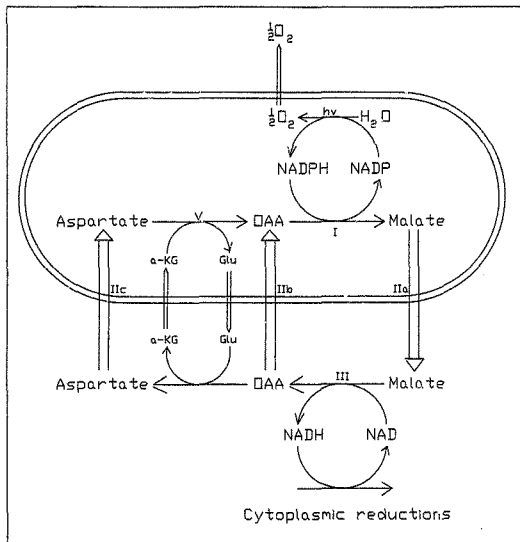


Fig. 2. The C_4 dicarboxylic acid shuttle for the export of light generated reducing equivalents from illuminated chloroplasts and their utilization in the reduction of a cytoplasmic substrates. Area within double lines represents chloroplast. Reaction I involves light coupled NADP-MDH with the concomitant evolution of O_2 . Reaction IIa involves the outward transport of malate with associated inward transport of OAA (reaction IIb) which is effected by the dicarboxylic acid translocator associated with the inner membrane. Reaction IIc is catalysed by NAD-MDH. Reaction IV can in theory be one of many reactions. Reaction III involves the inward transport of aspartate by the C_4 dicarboxylic acid translocator and transamination of OAA by chloroplast aspartate- α -KG amino transferase (reaction V) (After Anderson, 1981).

but not by NADH (Anderson and House, 1979). In C_4 plants the rôle of light coupled NADP-MDH is well established; it serves to reduce OAA to malate which is then transported to the bundle sheath cells where decarboxylation and recarboxylation through the C_3 cycle occurs (Anderson, 1981). The rôle of light coupled NADP-MDH in C_3 plants is less clear. It has been proposed that the light generated reducing equivalents (in the form of malate) may be transported from the chloroplast into the cytoplasm where they may be used for various reductive reactions. In this regard the proposal by Neyra and Hageman (1978) and Sawhney et al. (1978a,b) that NO_3^- may be reduced using this reductant is of interest. The chloroplast envelope is freely permeable to malate via the dicarboxylate translocator and exogenous malate enhances the reduction of NO_3^- in leaf tissue. It has thus been proposed that light enhanced NO_3^- reduction is mediated through malate (Neyra and Hageman, 1978; Rathnam, 1978). According to this hypothesis malate produced in the light may be oxidised in the cytoplasm for the formation of NADH, and OAA produced in the process could be recycled into the chloroplast for further reduction. This shuttle can in theory provide reductant for any cytoplasmic reactions of which NO_3^- is one. A scheme of the metabolic pathways involved is shown in Fig. 2. Chloroplasts supplied with enzymes and substrates are capable of reducing these substrates in the light (Anderson, 1981). O_2 evolution and pyruvate reduction did not proceed in the absence of any one of OAA, NAD-MDH, LDH, NAD or pyruvate, but malate could substitute for OAA (Anderson and House, 1979). Direct evidence for this shuttle under normal physiological conditions is lacking (Anderson, 1981) although Neyra and Hageman (1976) have provided evidence for the operation of this pathway in maize. An extension of this C_4 cycle has been proposed and includes the formation of aspartate from OAA and the translocation of this intermediate across the chloroplast membrane (Walker, 1976).

7.7.3.2. The PGA/DHAP shuttle

Both PGA and DHAP are intermediates in the Calvin cycle and represent major export products of the chloroplast. Because of the

specificity of the phosphate translocator for divalent ions the trivalent PGA is not transported out of the chloroplast during the light reaction because of the pH. Thus DHAP is more readily exported even though the PGA concentration in the stroma may be ten times that of DHAP (Heber and Heldt, 1981). The reduction of PGA to DHAP within the chloroplast is light dependent and proceeds through the phosphorylation of PGA by ATP to form diPGA which is reduced by NADPH to GAP with the concomittant release of Pi and GAP is converted to DHAP by an isomerase (Anderson, 1981). The DHAP is apparently more readily transported than GAP (Walker, 1976). DHAP is exported with the counter transport of PGA via the phosphate translocator and DHAP is oxidised in reactions involving enzymes of the glycolytic sequence with the release of NADH and ATP (Anderson, 1981). This shuttle can in theory provide reductant for any cytoplasmic reactions of which NO_3^- reduction is one. A scheme of the metabolic pathways involved is shown in Fig. 3. A variant of this pathway is the oxidation of GAP by an irreversible NADPH-GAPDH outside the chloroplast resulting in the formation of NADPH but not ATP (Anderson, 1981). The link between phosphate and NO_3^- may be more complex than the dependence of NO_3^- reduction on phosphate exchange across the chloroplastic membrane. Nitrate appears to regulate the transport of phosphate into the vacuole from the cytoplasm and may thus control the cytoplasmic levels of phosphate (Lamaze et al., 1984). The effect of NO_3^- on phosphate uptake into the tonoplast may result from the inhibition of the tonoplast ATPase by NO_3^- which has been reported by Lew and Spanswick (1985). This may have important implications for the effects of NO_3^- on carbon metabolism because of the rôle played by phosphate in the translocation of photosynthetic carbon products out of the chloroplast.

The principal difference between the dicarboxylate shuttle and the PGA/DHAP shuttle is that the latter involves the loss of reducing potential and phosphorylation potential from the Calvin cycle whereas the former involves only the withdrawal of reducing potential from the light reaction. This has important consequences for the effect of NO_3^- reduction on the assimilation of carbon dioxide. These pathways represent the possibilities for withdrawal of reducing potential from the light reaction. Other

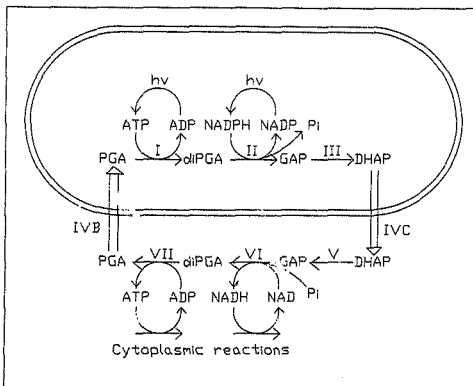


Fig. 3. The PGA/DHAP shuttle for the export of light-generated reducing equivalents and high-energy phosphate from illuminated chloroplasts. Reaction I to III involve enzymes of reductive CO_2 assimilation. DHAP is exported from the chloroplast (reaction IVa) with the counter transport of PGA (reaction IVb) via the phosphate translocator. The PGA and DHAP form part of the Calvin cycle intermediates pool. DHAP is oxidised in the cytoplasm in reactions involving enzymes of the glycolytic sequence (reaction V to VII) (After Anderson, 1981).

possibilities, which have received rather less attention may include the export of glucose, the participation of fatty acids and amino acids, although these mechanisms are likely to be less direct. The situation in roots or leaf tissue kept in the dark is considerably different and depends on the metabolism of storage products. Because roots are dependent on the supply of sugars from the shoot for growth, and considering the carbon requirements of NR, it is evident that NO_3^- could be an effective competitor for carbon. The effects of endogenous sugar levels on root growth and NO_3^- reduction have been shown to be different. Nitrate reduction was more adversely affected than root growth at low endogenous sugar levels (Radin et al., 1978). The dependence of NO_3^- assimilation in the root on the provision of photosynthate is illustrated by the proportion of NO_3^- reduced in dark and light conditions in the root. In the dark 40% of NO_3^- was reduced in the root whereas in the light only 20% was reduced here (Aslam and Huffaker, 1982). This also indicates the greater capacity of the leaves for NO_3^- reduction. Rufty et al. (1984) showed that uptake of NO_3^- in the dark was only slightly depressed (Dark 7.93 and light 8.42 $\mu\text{moles/hr}$) but total plant NO_3^- reduction was significantly diminished in the dark.

7.7.3.3. Carbon assimilation coupled nitrate reduction

Roots are known to assimilate NO_3^- in the dark by making use of photosynthates translocated from the green tissues. This would appear to indicate that NO_3^- assimilation is only indirectly dependent on photosynthetic carbon assimilation. However, light has often been shown to accelerate NO_3^- reduction. There are a number of proposals as to why light is required:

- light stimulates uptake of NO_3^-
- light promotes transfer of NO_3^- from storage to the metabolic pool
- light induces NR synthase
- light activates pre-existing NR
- photosynthesis provides reductant for NO_3^- assimilation (Naik et al., 1982).

Recently it has been claimed that the rôle of light is only to supply photosynthates for NO_3^- assimilation. That the in vivo assay for NR depends on dark anaerobic conditions for the reduction of NO_3^- further supported the view that NO_3^- reduction is essentially light independent. Calvin and Atkins (1974) concluded from experiments using $^{15}\text{NO}_3^-$ (supplied to leaves either through the transpirational stream or through vacuum infiltration) incorporation into amino acids that NO_3^- reduction under physiological conditions had an absolute requirement for light. In these experiments $^{15}\text{NH}_4^+$ was incorporated in the dark but light stimulated this incorporation 5-fold. From the data of these experiments it does however appear that there was some small incorporation of $^{15}\text{NO}_3^-$ into amino acids in the dark. Dark utilization of NO_3^- , even in chlorophyll free mutants of Oryza sativa has been reported (Yoneyama, 1984).

By adding various metabolites of the glycolytic, pentose phosphate and citric acid pathways, Klepper et al. (1971) demonstrated that sugars that migrated from the chloroplast were the prime source of energy and that GAP was the ultimate in vivo source of NADH for NR. Isolated leaf protoplasts of spinach are capable of NO_3^- reduction at rates of $9 \mu\text{moles.mg}^{-1}.\text{hr}^{-1}$ in the light with a 3-4 fold stimulation in the presence of HCO_3^- (Rathnam, 1978). In addition a light independent stimulation of NO_3^- reduction by malate and DHAP and a light dependent stimulation with OAA and PGA was demonstrated and these effects were attributed to the operation of the dicarboxylate and the PGA/DHAP shuttles (Rathnam, 1978; Mann et al., 1978). Woo and Calvin (1980) provided evidence that malate oxidation in the cytoplasm, above the reduction of NAD by malate in the mitochondrion, could account for the reduction of NO_3^- . Illuminated pea chloroplasts have been shown to reduce OAA to malate with the concomittant production of O_2 and in the presence of NAD, NAD-MDH and catalytic amounts of OAA, chloroplasts were capable of NO_3^- reduction (House and Anderson, 1980). In addition the enzyme NAD-MDH is light activated and it follows that the effect of light on NO_3^- reduction could be mediated both through the effect of light on this enzyme and through the supply of NADPH by the light reaction. The C_3 shuttle

could be similarly regulated because glyceraldehyde 3-phosphate dehydrogenase is also light activated (House and Anderson, 1980). In the absence of added PGA, DHAP or C_4 acids, glyceraldehyde (which inhibits the Calvin cycle) inhibits both CO_2 fixation and NO_3^- reduction by spinach protoplasts (Rathnam, 1978). This indicates that the physiological mechanism, at least in spinach, is through the DHAP/PGA shuttle.

The functioning of the DHAP/PGA translocator and the apparent dependence of NO_3^- reduction in the light has important implications for the effects of nitrogen on the partitioning of carbon from photosynthesis into starch and sucrose. Larsson et al. (1985b) reported that NO_3^- uptake-reduction was stimulated by CO_2 at high light intensities. In addition these authors show a competitive relationship between NO_3^- and CO_2 at low light intensities but this effect disappears at high light intensities. This was taken by the authors to imply that photoproduced reductant transferred across the chloroplast envelope was involved in NO_3^- reduction and depletion of this within the chloroplast resulted in inhibition of CO_2 fixation at low light intensities. Di Martino Rigano et al. (1985) found that CO_2 deprivation prevented the assimilation of NO_3^- by Chlorella vulgaris in a pH dependent fashion. At pH 6.5 no NO_3^- assimilation occurred whereas at pH 8.2 NO_3^- was reduced to NO_2^- but no further reduction occurred. In addition cells grown without phosphate were unable to assimilate NO_3^- even in 5% CO_2 . Under low nitrogen and phosphorus conditions starch has been shown to accumulate in Panicum maximum (Ariovich and Cresswell, 1983). These authors and others (Champigny, 1985) propose that this accumulation is the result of a reduced translocation of Calvin cycle intermediates out of the chloroplast. Leaf extracts of NO_3^- supplied plants have been reported to have higher SPE activity correlated with reduced starch accumulation (Kerr et al., 1984; Huber et al., 1985). Similar results were not obtained with plants dependent on sources of nitrogen other than NO_3^- (Kerr et al., 1984). Blackwood and Mifflin (1976) found that NO_3^- supplied to maize leaves increased the incorporation of ^{14}C into malate and aspartate, but decreased incorporation into sucrose and starch. Apart from the importance of partitioning of carbon into sucrose, the lack of

starch accumulation under NO_3^- supply is very significant. Starch is known to disrupt chloroplastic structure and function (Ariovitch and Cresswell, 1983).

Considering the importance attached to the formation of sucrose and starch from photosynthate it may be pertinent to mention the possibility of control through fructose 2,6-bisphosphate which exerts a powerful control over cytosolic fructose 1,6-bisphosphatase which is attributed a crucial rôle in controlling carbon assimilation (Champigny, 1985). This enzyme catalyses the first step in the formation of sucrose in the cytosol from triose-P. The absence of sufficient P_i results in a depression of photosynthesis as a result of the accumulation of phosphorylated intermediates in the stroma while excess P_i results in the withdrawal of TP so rapidly that RuBP is depleted and photosynthesis decreased (Stitt and Heldt, 1985). After export from the chloroplast, TP may be metabolised to sucrose with the concomitant release of P_i (Stitt and Heldt, 1985). Thus control of sucrose synthesis is important for the control of cytoplasmic P_i levels (Champigny, 1985). In addition, during rapid photosynthesis, excess sucrose may accumulate in which case starch is formed. Fructose 6-P, P_i , DHAP and PGA can control the synthesis and degradation of fructose 2,6- P_2 which in turn modulates the activity of fructose 1,6-bisphosphatase. There is also the possibility of direct effects of fructose 1,6- P_2 , DHAP, fructose 6-P, P_i , AMP, UDP, pH and Mg^{2+} on the activity of fructose 1,6-bisphosphatase (Stitt and Heldt, 1985).

Not all workers are in agreement that NO_3^- has a positive influence on photosynthetic CO_2 fixation and suppression of photosynthesis in Selenastrum minutum resulted from the supply of NO_3^- to previously nitrogen limited cells (Elrifí and Turpin, 1986). Supply of up to $100 \mu\text{M}$ NO_3^- resulted in a 70 % reduction in carbon assimilation and a 24 % reduction in oxygen evolution. On the basis of these results, and considering that similar supply to N sufficient cultures did not produce the same results it was concluded that the response was not the result of NH_4^+ uncoupling effects (Elrifí and Turpin, 1986). It was proposed instead that the inhibition resulted from competition between the

Calvin cycle and nitrogen assimilation with the TCA cycle supplying the carbon skeletons for nitrogen assimilation and the TCA cycle in turn reliant on the Calvin cycle. This was suggested to result in the depletion of RuBP thus reducing the efficiency of the Calvin cycle. These authors indicate a dependence of NH_4^+ assimilation on the provision of α -ketoglutarate from the TCA cycle but apparently ignore the possibility of the reliance of cytoplasmic NO_3^- reduction on either the respiratory pathways or the Calvin cycle, although it is proposed that starch and sucrose may play an important rôle as a source of TP for nitrogen assimilation.

7.7.3.4. The interdependence of photosynthetic and respiratory carbon metabolism and nitrate assimilation

Inhibition of photosynthetic electron transport by DCMU resulted in a decrease in the fixation of $^{14}\text{CO}_2$ and the reduction of $^{15}\text{NO}_3^-$ although $^{15}\text{NO}_3^-$ reduction was not as strongly inhibited as $^{14}\text{CO}_2$ fixation (Atkins and Canvin, 1975). Similar results were obtained with the uncoupler of photophosphorylation CCCP (Atkins and Canvin, 1975). These results may be interpreted as meaning that NO_3^- reduction is closely linked to the light reaction. Nitrate is however reduced in the dark but apparently only under anaerobic conditions. O_2 concentrations of 2% support only 25% of anaerobic NO_3^- reduction (Canvin and Woo, 1979). Inhibitors of mitochondrial electron transport had similar consequences to the deprivation of O_2 implying that NO_3^- reduction is promoted when mitochondrial dehydrogenase activity is inactivated (Canvin and Woo, 1979).

That NO_3^- and NO_2^- are only assimilated under anaerobic conditions when deprived of light has recently been challenged by Ben-Shalom et al. (1983) who reported that NO_3^- and NO_2^- were reduced in both light and dark conditions. The use of uncouplers in this type of study may be questioned however, because the inhibitors may have different effects depending on the concentration at which they are supplied. Gray and Cresswell (1984) found that low concentrations of uncouplers resulted in a decrease on both NO_3^-

reduction and NO_2^- accumulation relative to higher concentrations of these uncouplers. This may be related to the stimulation of respiratory electron transport associated with low levels of uncoupler while higher levels actually inhibit electron transport. A stimulation of electron transport would deplete the supply of NADH derived from respiratory pathways and thus inhibit the reduction of NO_3^- and the accumulation of NO_2^- . Gray and Cresswell (1984) and Ben-Shalom et al. (1983) report a decreased reduction of NO_3^- by plants supplied with light and oxygen on supply of DCMU. Other conditions (light + anaerobic + DCMU, dark + anaerobic + DCMU and dark + anaerobic + DCMU + CCCP) all stimulated NO_3^- reduction (Gray and Cresswell 1984). Similarly Ben-Shalom et al. (1983) and Reed et al. (1983) reported that conditions restricting mitochondrial electron transport stimulated NO_3^- reduction. This observation is supported by the small (2-3 fold) increase in NO_2^- assimilation under aerobic conditions compared to anaerobic conditions whereas NO_2^- accumulation under dark anaerobic conditions increased 2.5-20 fold over aerobic levels (Stitt et al., 1983). This indicates that NR is at least facultative in the use of photosynthetic and respiratory reducing potential. Inhibition of the light reaction with DCMU could result in the reduction of the supply of photosynthetically produced reducing potential while similar treatment in the dark could limit the functioning of oxidative phosphorylation and thus make NADH more available for NO_3^- reduction. Thus, under conditions limiting mitochondrial electron transport, anaerobic respiration could proceed or the glycolytic and TCA pathways may operate with the NADH generated from these two paths being oxidised by NO_3^- .

Although light has been implicated in the reduction of NO_3^- it has been suggested that NO_3^- reduction is essentially linked to respiration and that light really only plays a rôle in controlling the availability of respiratory NADH. In this regard Sawhney et al. (1978b) suggest that increased adenylate charge resulting from photophosphorylation inhibits mitochondrial respiration and that the reducing equivalents resulting from glycolysis and the TCA cycle may be diverted into NO_3^- reduction. This suggestion was based on the observation that infiltrated ATP and fructose-1-

6-diphosphate (which could be rapidly metabolised to yield NADH and ATP by glyceraldehyde-3-phosphate dehydrogenase) led to an accumulation of NO_2^- even under aerobic conditions. Thus ATP was suggested to prevent the dark accumulation of NO_2^- . Dry et al. (1981) have shown that at least for root plastids, NO_2^- reduction was dependent on the supply of glucose-6-phosphate, and did not require ATP. A negative correlation between NO_2^- accumulation in anaerobic conditions and the level of G6P led to the proposal by these authors that anaerobic metabolism of G6P depleted this metabolite leading to an accumulation of NO_2^- . G6P was proposed to be utilized via the pentose phosphate pathway for the provision of NADPH for NO_2^- reduction. Chloroplastic NADPH could reduce ferredoxin in the dark through the reversal of Fd:NADP oxidoreductase and the chloroplastic NADP may be imported via the malate/oxaloacetate, glucose-6-P/6-P-gluconate or the PGA/DHAP shunts (Stitt et al., 1983).

That photophosphorylation inhibits mitochondrial respiration is however equivocal. Reports in the literature indicate that respiration continues in the light while, on the other hand, light inhibition of respiration has been reported. Plant mitochondria, unlike animal mitochondria, are reported to be relatively insensitive to the extramitochondrial ATP/ADP ratio (Hamp, 1985). In contrast Tobin and Givan (1984) report extensive effects of exogenous ATP and ADP on plant mitochondrial malate oxidation. ATP was found to increase pyruvate production and decrease oxaloacetate formation independent of electron transport. Stitt et al. (1982) reported that cytosolic ATP/ADP ratios actually decreased in the light which contradicts the widespread assumption that export of photosynthetically produced ATP increases cytosolic ATP/ADP and results in a decrease of oxidative phosphorylation. For this reason other forms of control have been examined. During dark-light transition a transient increase in the mitochondrial NADH pool has been found to be correlated with an increase in the concentration of TP in the cytoplasm (Hamp, 1985). This initial inhibition of mitochondrial respiration was followed by a decrease in the extent of inhibition during steady state photosynthesis. This is attributable to the decline in the levels of TP (mostly in the

form of DHAP) from 30 mM in the cytosol to between 2 and 5 mM leading to only a moderate inhibition of mitochondrial respiration (Hamp, 1985). Thus mitochondrial respiration probably has a substantial rôle in the provision of ATP in the light.

7.7.3.5. Rôle of respiration in nitrate reduction

From the observations described in the previous sections, it can be concluded that under light conditions, NO_3^- reduction is facilitated by the export of TP and the utilization of this for the provision of reductant. In the dark under aerobic conditions O_2 competes strongly with NO_3^- for NADH, but any factor (anaerobic conditions or supply of inhibitors) which limits oxidative phosphorylation results in a stimulation of NO_3^- reduction through the removal of the competitive effects of O_2 (Radin, 1973; Hewitt et al., 1979). In spinach the concentrations of NADH were found to be 8 μM in dark aerobic tissue, 13 μM in dark anaerobic tissue and 17 μM in light aerobic tissue. The dark levels are 2-3 times the K_m of NR while the light levels are 4-5 times the K_m (Hewitt et al., 1979). These levels are for leaf tissue however and do not reflect the intracellular distribution of NADH. At very high levels of NO_3^- (100 mM) Radin (1973) found that strictly anaerobic conditions were not required for the dark reduction of NO_3^- . This may indicate that high NO_3^- levels may competitively overcome the inhibition of NO_3^- reduction by oxygen. The levels of NADH even under anaerobic conditions have been reported to be sufficient for the reduction of NO_3^- (Mann et al., 1979). These authors proposed that the effects of some of the inhibitors (DNP and CCCP) could be primarily on the membranes where they may control the flux of NO_3^- . Under physiological conditions, inhibition of mitochondrial oxidation of NADH is either brought about through enhanced cytoplasmic adenylate charge or the presence of triose-P.

The question now arises as to which respiratory pathways are responsible for the supply of reductant to NR. The requirement for anaerobic conditions immediately suggests the participation of anaerobic fermentation in this process. Under anaerobic

conditions, respiratory degradation of glucose results in the accumulation of alcohol and/or lactic acid. The pathway involves the normal glycolytic sequence up to the transport of pyruvate into the mitochondrion where it would normally be oxidised via the TCA cycle. Under anaerobic conditions this oxidation is not possible and an alternative system for the removal of pyruvate has to operate to avoid the accumulation of pyruvate (Lehninger, 1976). An additional problem is that the NADH formed during the oxidation of GAP cannot be reoxidized by oxygen to form ATP. One solution is the reduction of pyruvate to lactate catalysed by lactic acid dehydrogenase which converts NADH to NAD in the process. This occurs in some plant systems but declines after a few hours and only accounts for a small proportion of anaerobic respiration (Reggiani et al., 1985). The alternative is the decarboxylation of pyruvate to acetaldehyde, catalysed by pyruvic carboxylase and the subsequent reduction of acetaldehyde to ethanol by alcohol dehydrogenase with reoxidation of NADH. This pathway has often been implicated in the reduction of NO_3^- under anaerobic conditions. The operation of the anaerobic pathway in parallel to the reduction of NO_3^- implies that these two processes must be competitive for NADH. An alternative mechanism for the provision of reductant to NR under anaerobic conditions is discussed later.

Dry et al. (1981) have reported a decline in glucose 6-P correlated with increased NO_2^- production. The levels of glucose 6-P were suggested by these authors to be regulated by O_2 through ATP/ADP ratios and their effects on glycolysis. The utilization of glucose 6-P during anaerobiosis may deplete this metabolite thus preventing the utilization of this metabolite for the provision of reductant for NO_2^- reduction (Dry et al., 1981). Under anaerobic conditions ATP is thought to become limiting and this would relieve the regulatory effect of ATP on PFK, or indirectly through fructose 2,6 bisphosphate on PFK (Champigny, 1985), and result in the rapid metabolism of glucose. Because anaerobic respiration yields little ATP this system is thought to continue operating at a high rate. The proposal that anaerobic respiration could provide reductant for NO_3^- reduction implies that pyruvate or acetaldehyde must accumulate. For the reduction of 1 NO_3^- we

require the provision of 1 NADH which means that 1 pyruvate cannot be reduced to ethanol/lactic acid.

An additional consideration is that alcohol dehydrogenase may be an effective competitor for reductant with NR. Thus, although anaerobic respiration probably does occur under the appropriate conditions, it does not seem likely that it could be an effective source of reductant for NO_3^- reduction. An additional consideration is that the anaerobic stimulation of NO_3^- assimilation was not found in barley roots *in vivo* (Lee, 1978). Anoxia resulted in a decrease in the capacity of nitrate assimilation and, at least partial, inhibition of NO_2^- reduction (Lee, 1978). These results may in part be related to the observation that NO_3^- may be compartmentalised away from the metabolic pool and may be unavailable during anaerobic conditions (Lee, 1978; Ferrari et al., 1973). A similar reduction in the capacity for NO_3^- reduction under anaerobic conditions has been reported for barley leaf tissue by Soares et al. (1985a) but these authors reported that this could not be attributed to the compartmentation of the NO_3^- within the cell. These authors proposed a model in which NO_3^- is reduced to NO_2^- in a confined metabolic compartment from which NO_2^- cannot escape under conditions of low ATP.

Nitrate does however stimulate the anaerobic evolution of CO_2 and the accumulation of ethanol in rice roots. Under anaerobic conditions the ratio of ethanol/ CO_2 was not affected by the presence of NO_3^- while the adenylate energy charge was elevated by NO_3^- (Reggiani et al., 1985). The ratio of ethanol/ CO_2 was very high indicating the possible operation of other pathways, but NO_3^- does not influence the relative contributions of these pathways. The enhanced production of ethanol contradicts the assumption that NR and ADH compete for reductant (Reggiani et al., 1985). The two factors which prevent normal glycolytic respiration under anaerobic conditions are the accumulation of NADH and pyruvate (Bidwell, 1974). In the light there is likely to be an adequate supply of TP in the cytoplasm which could participate in the glycolytic sequence resulting in the formation of NADH. These substances are also likely to generate NADH in the mitochondrion through the TCA cycle (Ramarao et al., 1981). Pyruvate accumu-

lates because the TCA cycle cannot operate under these conditions as a result of the accumulation of NADH and FADH, which in turn cannot be reoxidized. It is well known that the malate-aspartate shunt is capable of transporting NADH into and out of the mitochondrion (Lehninger, 1976). Thus cytoplasmic NADH and mitochondrial NADH pools are in reality part of one respiratory NADH pool. If there was a mechanism for the oxidation of NADH then glycolysis and the TCA cycle could operate in the absence of oxygen. That NO_3^- could fulfill this requirement has been suggested by Naik and Nicholas (1981). These authors found a NO_3^- dependent evolution of $^{14}\text{CO}_2$ from leaf disks in anaerobic conditions supplied with ^{14}C labeled citric acid cycle intermediates. A 1:1 stoichiometry was observed between NO_2^- production and $^{14}\text{CO}_2$ evolution. Sawhney et al. (1978) observed that PEP, pyruvate and other citric acid cycle intermediates stimulated NO_3^- reduction and proposed that TCA produced NADH was important for NO_3^- reduction. Malate exported from the mitochondrion has also been proposed as a source of reductant for NR (Woo and Calvin, 1980). This malate may be directly exported from the TCA cycle or may function as a carrier for NADH. Malonate completely inhibits NO_3^- reduction in vivo under dark anaerobic conditions indicating that the citric acid cycle is essential to provide reductant and that glycolytic sources are not that important (Ramarao et al., 1981). In the light the TCA cycle may be slightly different to the normal pathway as a result of the import of malate which is rapidly produced by the chloroplast (Ramarao et al., 1981). Thus the proposal that the TCA cycle produces NADH which is exported from the mitochondrion by a malate-oxaloacetate shunt enjoys some experimental support.

An additional possibility is that the hexose-monophosphate shunt, which produces CO_2 and NADPH could be responsible for the reduction, at least in part, of NO_3^- . The NADPH may reduce NAD through the enzyme transhydrogenase and the NADH thus produced may participate in NO_3^- reduction (Dailey et al., 1982). Alternatively the NADPH may directly reduce the NADPH:NR which is found in some plant species at least as a constitutive enzyme (Robin et al., 1985). The HMP pathway also produces GAP, but this may be combined with DHAP (produced from GAP by an isomerase) to form

F6P which can then re-enter the pathway. The operation of this pathway in the cytoplasm normally accounts for a significant proportion of glucose degradation and may be favored during NO_3^- reduction. NADPH has been shown to act as the physiological reductant of NO_3^- under dark aerobic conditions (Ramarao et al., 1981). The activity of one of the primary enzymes of the hexose-monophosphate shunt (glucose 6-P dehydrogenase) in Chlamydomonas reinhardtii has been reported to increase in the presence of NO_3^- as compared to NH_4^+ fed cells (Hipkin and Cannons, 1985). Thus there is evidence for the involvement of this pathway.

Chloroplast respiration describes the operation of oxidative phosphorylation and glycolysis (starch to PGA) within the chloroplast (Kow et al., 1982). Originally it was supposed that the chloroplast derived reducing potential and ATP from the cytoplasm in the dark. Considering the low permeability of the chloroplast membrane to NAD(P)H and ATP it seems unlikely that this would be the case, although the dicarboxylate and PGA/DHAP shuttles could fill this requirement to some extent. Kow et al. (1982) reported the oxidation of glyceraldehyde-3-P to PGA with the formation of NADP which could be converted to $\text{Fd}(\text{rd})$. The carbon source was suggested to be chloroplast starch. The reductant provided by such reactions was suggested to be utilized for a number of reactions including NO_2^- reduction to amino acids.

Nitrate and NH_4^+ absorption by roots elicited an immediate respiratory rise of 160 % in roots of Pisum sativum, 90 % of which was attributable to an alternative respiratory path not involving cytochrome c (DeVisser et al., 1986). This was not accompanied by an immediate increase in shoot photosynthesis although shoot photosynthesis did increase after a 1 hour delay indicating that the respiratory effect was not photosynthesis related.

7.8. THE REDUCTION OF NITRITE

Nitrite, the product of NO_3^- reduction, must be further reduced to NH_4^+ before the constituent nitrogen can enter into organic combinations. Nitrite rarely accumulates and has been reported to be toxic (Hewitt et al., 1976). Nitrite reduction involves the transfer of six electrons.



Nitrite reductase is a single protein enzyme with a molecular weight of 62 000 (Hewitt et al., 1976) although a molecular weight of 68 000 has been reported (Ishiyama et al., 1985). The enzyme possesses one sirohaeme which functions in the transfer of the six electrons. The enzyme is thought to occur mainly in the chloroplasts in leaves and in plastids in roots (Magalhaes et al., 1974; Neyra and Hageman, 1974; Miflin, 1974; Plaut et al., 1977). Other workers have proposed that NfR occurs in the cytoplasm (Grant et al., 1970) or in the peroxisome (Lips and Avissar, 1972). The enzyme from a variety of sources (Phaseolus vulgaris green shoots, etiolated shoots and roots) was found to have common antigenic determinants (Ishiyama et al., 1985). Illuminated intact chloroplasts reduce NO_2^- rapidly without any additional requirements. Spinach chloroplasts in the light actively reduce NO_2^- of which 60-90% is incorporated into amino acids (Anderson and Done, 1978). Nitrite reduction was found to be functionally associated with electron transport arising from the light reactions of the chloroplast. Nitrite reductase was reported to be closely associated with photosystem I and ATP was found to have no direct effects on the enzyme (Neyra and Hageman, 1974). In contrast Sawhney et al. (1978a) report no effect of ADP and Pi on the reductive assimilation of NO_2^- while ATP was reported to promote NO_2^- accumulation. Initially the reduction of NO_2^- to NH_4^+ was proposed to involve the production of free intermediates.



Hyponitrite and hydroxylamine have not been demonstrated to exist in any appreciable amounts in plant tissue and the former is not reduced by NiR (Hewitt et al., 1975). It is now believed that the reduction of NO_2^- to NH_4^+ does not involve the formation of free intermediates, but that the intermediates, if they exist at all, remain bound to the enzyme complex.

The NiR enzyme is inducible in the presence of both NO_3^- and NO_2^- . Recent studies have shown that light and NO_3^- modulate NiR levels (Gupta and Beevers, 1985). Use of antibodies against NiR was made to determine whether the synthesis of this enzyme is regulated at the level of transcription or translation (Gupta and Beevers, 1985). From these studies it was concluded that NO_3^- is required for the formation of NiR either in dark or light conditions and that synthesis is stimulated by light. In addition it appears that NO_3^- regulates transcription while light may control either transcription or translation. The induction of NiR may be enhanced in the presence of either NO_2^- or NO_3^- , by exogenous sucrose, glucose and fructose and other hexoses, although the induction was more marked in the presence of NO_3^- (Sahulka, 1981). The reduction of NO_2^- is associated with O_2 evolution only in chloroplasts isolated from plants previously supplied with NO_3^- . This is consistent with the induction of NiR by NO_3^- (Anderson and Done, 1978).

7.8.0.1. Reduction requirements of Nitrite Reductase

In initial studies benzyl viologen and methyl viologen was used as an artificial electron donor but subsequently $\text{Fd}_{(\text{rd})}$ has been shown to be capable of providing the reducing power. Initially the enzyme was shown to have NADPH dependent activity. Purified NADPH dependent NiR has been shown to separate into two fractions; a Fd -NADP reductase activity capable of transferring electrons from NADPH to $\text{Fd}_{(\text{ox})}$ and a NiR component capable of accepting electrons from $\text{Fd}_{(\text{rd})}$ and a K_m of 0.15 mM for NO_2^- (Joy and Hageman, 1966). The purified NO_2^- reductase was proposed to accept electrons directly from benzyl viologen and $\text{Fd}_{(\text{rd})}$ while

NADPH and dithionite electrons were channeled through Fd (Joy and Hageman, 1966).

Although NiR and GOGAT (see below) are found in roots and are active with Fd_(rd) prepared from spinach leaf material, a natural electron donor in roots has only recently been characterized. A non-haeme iron containing protein, with antigenic similarity to ferredoxin has been identified in maize roots (Suzuki et al., 1985). In addition a pyridine nucleotide reductase immunologically similar to Fd-NADP reductase from spinach leaves has also been characterized by these workers. Under chemically reducing conditions these carriers served as electron carriers for maize root GOGAT and NiR. NADPH could drive GOGAT using these carriers but not NiR (Suzuki et al., 1985). The question arises as to what the physiological reductant for NO₂⁻ reduction is in the root plastid. The reductant has been proposed to be derived from the pentose phosphate shunt. Glucose 6-P or 6-phosphogluconate is proposed to reduce NADP which then passes the reducing potential through a diaphorase to a carrier (suggested to be that proposed by Suzuki et al. (1985)) and onto NO₂⁻ (Oji et al., 1985). These authors cite as evidence for this pathway the presence of the required enzymes in the root plastid.

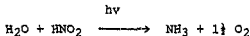
7.8.0.2. The source of reductant for Nitrite Reductase

The chloroplastic location of the NiR enzyme is strong evidence for the dependence of the enzyme on the photosynthetic provision of reducing equivalents. Nitrite assimilation of chloroplasts from a number of crops is linked to the availability of Fd_(rd) generated through photosynthetic electron transport reactions (Venkataramana and Das, 1983). Photoreduction of NO₂⁻ in the stroma of higher plant chloroplasts involves the participation of Fd_(rd). Ferredoxin is required for both the reduction of NO₂⁻ and assimilation of NH₄⁺; considering the total flux of NO₂⁻ and NH₄⁺ the supply of Fd_(rd) needs to be considerable. This is especially true when the flux of NH₄⁺ from photorespiration is considered. The assimilation of NO₂⁻ to glutamate requires 8 Fd_(rd) and 1 ATP (Robinson, 1986). The Fd_(rd) employed for NO₂⁻ assimilation may

alternatively be routed through NADPH to the Calvin cycle for the photoassimilation of CO_2 . The magnitude of CO_2 assimilation is 5-20 times greater than the magnitude of NO_2^- reduction.

Light dependent assimilation of NO_2^- and CO_2 were equally inhibited by DCMU and similarly inhibited by CCCP (Atkins and Calvin, 1975). This is not consistent with other reports indicating that CO_2 assimilation was considerably more sensitive to DCMU than NO_2^- (Mifflin, 1974). These results do however indicate that NO_2^- and CO_2 assimilation are closely linked to photosynthetic electron transport.

The O_2 evolution dependence of NO_2^- reduction may be summarized as:



This equation is derived from the fact that the photosynthetic electron transport system produces 4 electrons for the formation of 1 O_2 and the reduction of NO_2^- requires 6 electrons. The O_2/NO_2^- ratio approached 1.5 in short term experiments, but on the whole was lower (Anderson and Done, 1978). Mifflin (1974), working with isolated spinach chloroplasts, reported ratios of 1.15 for the first 30 minutes of reduction, 1.45 for the second thirty minutes and 1.86 for the third period. This deviation was attributed by this author to the inhibitory effects of NO_2^- in O_2 evolution which were reported by Grant et al. (1970). These experiments were performed in CO_2 free media and, although Mifflin (1974) did report inhibition of O_2 evolution by NO_2^- (0.4 mM) over long time periods (53 minutes), there was also a NO_2^- dependent O_2 evolution component of his results over a shorter term (20 minutes).

The rate at which NO_2^- reduction occurs falls in the range 10 - 25 $\mu\text{mol NO}_2^-\cdot\text{h}^{-1}\cdot\text{mg chl}^{-1}$ which would indicate a maximum demand for Fd(rd) of 200 $\mu\text{mol}\cdot\text{h}^{-1}\cdot\text{mg chl}^{-1}$ (Robinson, 1986). CO_2 assimilation has been suggested to repress NO_2^- reduction indicat-

ing some competition between the two processes for Fd(rd) (Larsson et al., 1985b) although other workers have reported no effect of CO₂ assimilation on NO₂⁻ reduction. Supply of DL glyceraldehyde, which inhibits the Calvin cycle, has also been found to stimulate NO₂⁻ reduction (Anderson and Done, 1978). Supply of NO₃⁻ (Robinson, 1986) and NH₄⁺ (Woo and Calvin, 1980) have been reported to stimulate photosynthetic CO₂ assimilation. The interdependence of CO₂ fixation and NO₂⁻ assimilation has been further demonstrated through the observation that a stimulation of NO₂⁻ reduction may be achieved by allowing isolated chloroplasts a short period of photosynthetic CO₂ fixation in the light prior to the addition of NO₂⁻. A similar response was achieved through the addition of DHAP or fructose 6-P and the suggestion was made that photosynthetic intermediates may regulate NO₂⁻ reduction (Plaut et al., 1977).

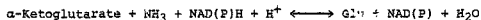
Measurements of Fd indicate that its tissue concentrations is 2mM which, at high light intensity, should provide adequate reduced Fd to support both CO₂ assimilation and NO₂⁻ reduction without competition (Buchanan, 1980). Baysdorfer and Robinson (1985) report a decline of NADP reduction rates on supply of NO₂⁻ which implies that NO₂⁻ can successfully compete for Fd(rd) even at high rates of NADP reduction. In contrast Robinson (1986) reported equal rates of NO₂⁻ reduction by spinach and soybean at CO₂ concentration in the range 0.5 mM to 5 mM. In addition the same author reported a stimulation of CO₂ assimilation by plastids in the range 50 μM - 100 μM NO₂⁻ while leaf mesophyll cells responded positively to NO₂⁻ between 250 μM and 2500 μM. These findings led to the conclusion that NO₂⁻ and CO₂ photosynsimilatory processes do not inter-compete at high light intensity (1000 μE.m⁻².s⁻¹). Depression of NO₂⁻ uptake and reduction has been reported in Scenedesmus during CO₂ fixation (Larsson et al., 1985b). Stimulation of O₂ assimilation by NO₂⁻ was not found in protoplasts or chloroplasts of spinach by Kaiser and Heber (1983). These authors used O₂ as an indicator of photosynthetic activity but it should be noted that O₂ evolution and CO₂ fixation are in reality quite distinct. In contrast to the findings of Robinson (1986), Purczeldt et al. (1978) reported an inhibition of CO₂ fixation in intact spinach chloroplasts at pH 7.3 in

presence of 1 mM NO_2^- . This effect was attributed to stromal acidification which inhibited the activity of fructose and sedoheptulose biphosphatase which play an important rôle in the regulation of CO_2 fixation (Purczeldt et al., 1978; Kaiser and Heber, 1983). Similar reasoning was invoked by Larsson et al. (1985b) to explain the inhibitory effects of NO_2^- on CO_2 assimilation in *Scenedesmus*. Thus the effects of NO_2^- on photosynthesis are complex and not attributable to any one phenomenon.

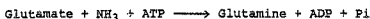
7.9. THE REDUCTION OF AMMONIUM

Two possible routes of NH_4^+ assimilation have been postulated:

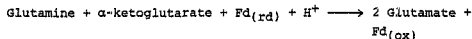
Route 1: The reductive amination of a keto acid to yield an amino acid directly catalysed by the enzyme glutamate dehydrogenase.



Route 2: The initial incorporation of NH_4^+ into glutamine by glutamine synthetase.



The amine group of glutamine is then transferred to the α position of α -ketoglutarate by the enzyme glutamine synthase NAD(P)H oxidising or $\text{Fd}_{(\text{rd})}$ oxidising, otherwise named glutamine: α -ketoglutarate amino transferase (GOGAT).



The metabolism via both routes requires a minimum of two reducing equivalents plus an additional ATP in route 2. In addition carbon is required for the formation of glutamate (5 carbon atoms).

(Lea and Miflin, 1979)

Ammonium assimilation in higher plants was long thought to begin with the synthesis of glutamate by glutamate dehydrogenase. It is now believed that the major pathway for NH_4^+ assimilation is the glutamine synthetase-glutamate synthase pathway. Supporting evidence for this pathway has been produced from ^{15}N kinetic studies showing that NH_3 is first assimilated into glutamine and then into glutamate (Kaiser and Lewis, 1980; Klaus et al., 1985). In tobacco cell cultures it was concluded from $[^{13}\text{N}]$ ammonium and $[^{13}\text{N}]$ nitrate experiments that the major route for the assimilation of NH_4^+ is through the glutamine synthetase-glutamate synthase pathway although a minor contribution was made by glutamate dehydrogenase (Skokut et al., 1978). Similar results have been reported for maize roots and leaves (Easkakova and Izmailov, 1984) and barley roots (Lewis et al., 1983). Response of chloroplasts to low NH_4^+ concentrations (10^{-4} M) indicate that GS-GOGAT is the pathway for NH_4^+ assimilation in this organelle.

Glutamine synthetase has been found in several plant tissues and was observed to be localized in the chloroplast and the cytoplasm (Lea and Miflin, 1979). GS has an extremely low K_m of 19 μM which means that cellular NH_4^+ could be kept at very low levels (100 μM) thus preventing any toxic effects. The activity of this enzyme is stimulated by light (Lea and Miflin, 1979). NADH dependent GOGAT and $\text{Fd}(\text{rd})$ dependent GOGAT have been extracted from several plant tissues but also appears to be associated with the chloroplast or root plastid. In higher plant leaves glutamate synthase is $\text{Fd}(\text{rd})$ dependent (Klaus et al., 1985) although roots and cell cultures may have the pyridine nucleotide dependent form. The enzyme is specific for glutamine with a K_m of 330 μM and can only function with α -ketoglutarate as an acceptor (Lea and Miflin, 1979).

Glutamate dehydrogenase exhibits a 10 fold higher activity with NADH than with NADPH. The levels of glutamate dehydrogenase in plant tissue are usually low and are associated with the mitochondrion with only extremely low levels detectable within the chloroplast (Lea and Miflin, 1979). Both NADPH and NADH forms of glutamate dehydrogenase have been described for chloroplasts. The enzyme shows a greater NADH activity but a chloroplastic

NADPH activity has also been reported with a K_m for NH_4^+ of 5.8 mM. Such a high K_m makes it unlikely that this enzyme could function in vivo (Lea and Miflin, 1979).

Although glutamate dehydrogenase is now attributed a relatively minor rôle in NH_4^+ assimilation, some authors still claim this enzyme to have an important rôle in inorganic nitrogen assimilation. The glutamate dehydrogenase enzyme has been found to have several isozymes which are differentially sensitive to specific nitrogen sources (Loyola-Vargas and De Jiménez, 1984).

7.9.1 The source of reductant for ammonium assimilation

In barley leaves the conversion of inorganic nitrogen to amino acids was found to be stimulated in the presence of light. In the presence of NH_4^+ , freshly assimilated CO_2 is withdrawn from organic acids and carbohydrates and is recovered in amino acids (Klaus et al., 1985). With supply of NH_4^+ increased amounts of $^{14}\text{CO}_2$ are incorporated into amino acids (Platt et al., 1977). Thus supply of NH_4^+ may be expected to alter the partitioning of photosynthate into sucrose and starch. Alfalfa leaf disks supplied with NH_4Cl and $^{14}\text{CO}_2$ produced more labeled amino acids and less sucrose than control leaf disks (Platt et al., 1977). These changes were accompanied by an increase in pyruvate and a decrease in PEP which led to the conclusion that the activity of pyruvate kinase was increased by NH_4^+ (Platt et al., 1977). Pyruvate, derived from PEP, is presumably metabolised to α -ketoglutarate which in turn may form the carbon skeletons for glutamate and glutamine. The formation of these amino acids suggests that OAA was formed and utilised at an increased rate. OAA may be derived from cytoplasmic PEP carboxylase or from mitochondrial oxidation of malate. Lea and Miflin (1979) concluded that there was little evidence to suggest that chloroplasts can independently synthesize the carbon skeletons for NH_3 assimilation. It is more likely that carbon leaves the chloroplast as DHAP and PGA and is converted to α -ketoacids in the mitochondrion and cytoplasm before re-entry into the chloroplast. This suggestion is not in accord with the findings

of other authors. The suggested interaction of exogenous NH_4^+ with cytoplasmic and mitochondrial metabolites implies that the NH_4^+ must exert its influence in these compartments from within the chloroplast. Exogenously supplied NO_3^- has been reported to result in the activation of pyruvate kinase and amino acid levels. Thus it is possible that chloroplastic NH_4^+ penetrates the chloroplastic membrane in order to exert an effect on the cytoplasmic and mitochondrial compartments (Platt et al., 1977).

Ammonium derived from NO_2^- is thought to be incorporated into amino acids within the chloroplast and is thus reliant on concurrent photosynthesis for the provision of carbon skeletons. While a proportion (10%) of photosynthate had been traced into amino acids it is likely that stored carbon would be required to meet the demand for carbon during rapid NH_4^+ assimilation (Atkins and Canvin, 1975). In isolated chloroplasts the conversion of glutamate to glutamine occurs only in the light. In low light, the lack of ATP restricts glutamine synthetase activity in low light (Klaus et al., 1985). Labeling patterns of glutamine and glutamate from supply of $^{15}\text{NH}_4^+$ to maize indicate glutamate synthase is active under both high and low light conditions while glutamine synthetase appears to be inhibited under low light conditions (Klaus et al., 1985). The net uptake of $^{15}\text{NH}_4^+$ into intermediates of the photorespiratory cycle (glycine) was rapid under high light conditions while low light inhibited this process. Under low light conditions maize incorporated only 40% of supplied $^{15}\text{NH}_4\text{Cl}$ into organic matter while at high light this increased to 92% (Klaus et al., 1985). These authors concluded that light influences NH_4^+ assimilation at the glutamine synthetase reaction (Klaus et al., 1985).

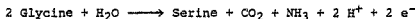
Ammonium assimilation exhibited quite different responses to the inhibitors DCMU and CCCP (supplied to barley) in comparison to the responses of NO_2^- and NO_3^- (Atkins and Canvin, 1975). There appeared to be a light dependent portion of NH_4^+ assimilation which was subject to inhibition but also a light independent fraction (Atkins and Canvin, 1975). In addition the DCMU inhibition of CO_2 assimilation by 92% was accompanied by a 75% inhibition of NH_4^+ assimilation. The smaller reduction of NH_4^+ as-

similation in the presence of the inhibitors may be attributable to a light independent assimilation of NH_4^+ . At low concentrations of CCCP NH_4^+ assimilation was observed to be stimulated, possibly as a result of uncoupling of oxidative phosphorylation leading to greater availability of carbon skeletons and reductant (Atkins and Calvin, 1975). Correlative evidence for the interdependence of NH_4^+ and CO_2 assimilation may be derived from the work of Larsson et al. (1985b). Ammonium supply to Scenedesmus resulted in the inhibition of CO_2 assimilation independently of light intensity. This inhibition of CO_2 assimilation is contrary to other reports of stimulation by Engemann and Brown (1980) who suggested that cytoplasmic acidification induced by NH_4^+ stimulated light and dark PEPc activity in Avena coleoptiles. The inhibitory influence was accompanied by a drop in ATP levels attributed to partial uncoupling. These authors also found evidence for the control of NH_4^+ assimilation by CO_2 fixation but did not consider the mechanisms of such effects.

Ammonium plus α -ketoglutarate dependent O_2 evolution attributable to the operation of the GS-GOGAT system for NH_4^+ assimilation into glutamate catalysed by glutamine synthetase and glutamate synthase was found by Anderson and Walker (1983). This reaction was dependent on a supply of Fd, glutamate and ADP (or ATP) in addition to NH_4^+ and α -ketoglutarate. Reconstituted chloroplasts required the provision of glutamate at high levels for this activity to become manifest while intact chloroplast required no addition of glutamate, presumably as a result of high endogenous glutamate levels. The $(\text{NH}_3$ plus α -ketoglutarate)-dependent O_2 evolution was found to be enhanced 3-5 fold by 2mM L- and D-malate. Although malate has been suggested to facilitate transport of α -ketoglutarate into the chloroplast via the dicarboxylate shuttle, this has been questioned by Anderson and Walker (1983) who found that α -ketoglutarate was taken up at rates saturating GS-GOGAT. No evidence for direct effects of NH_4^+ were found. This reaction was attributed to primary and photorespiratory assimilation of NH_4^+ linked to the light reaction for provision of ATP, Fd_(rd) and carbon (as α -ketoglutarate) directly or indirectly derived from the Calvin cycle.

7.10. THE ROLE OF INORGANIC NITROGEN IONS IN PHOTORESPIRATION

During photorespiration, glycine is converted to serine in the mitochondrion in an ATP liberating step which results in the release of NH_3 in stoichiometric quantities to CO_2 evolved from photorespiration (Keys et al., 1978).



With significant photorespiratory activity (up to 20% primary CO_2 fixation), the NH_3 produced would have to be assimilated at rates considerably higher than required from NO_3^- reduction. The NH_3 liberated in the mitochondrion may be refixed either in the cytoplasm or the chloroplast.

Evidence for the existence of an alternative pathway for photorespiratory decarboxylation involving the oxidation by H_2O_2 of glycolate to CO_2 and formate has been obtained from studies using MSO (Amory and Cresswell, 1986). In the presence of this inhibitor of glutamine synthetase, and also in the presence of NO_3^- or NH_4^+ , increases in CO_2 compensation point of Themeda triandra correlated with formate pool size (Amory and Cresswell, 1986). This pathway was suggested by these authors to improve nitrogen use efficiency, but to concomitantly reduce productivity by increasing photorespiratory CO_2 disassimilation.

Competition between CO_2 and O_2 at RuBPC/o results in the production of both 3-phosphoglycerate and glycolate, the latter of which is metabolised via a series of reactions in the chloroplast, peroxisomes and mitochondria with the evolution of CO_2 (Wallsgrave et al., 1983). This reaction is vital to the understanding of the fixation of CO_2 and the reduction of nitrogen because large amounts of both carbon and nitrogen pass through this pathway. The release of CO_2 occurs in the mitochondrion during the oxidation of glycine to serine by glycine decarboxylase with stoichiometric release of NH_3 which may be reassimilated in the chloroplast or cytoplasm by glutamine synthetase (GS) and glutamate synthase with the participation of NAD(P)H and/or Fd (Wallsgrave et al., 1983). If GS is inactivated by MSO,

NH_3 accumulates, but this accumulation may be abolished through the addition of exogenous GS (Wallsgrove et al., 1983). Martin et al. (1983b) reported an accumulation of NH_3 to 16% of the apparent CO_2 assimilation rate in C_3 leaves exposed to MSO. From these experiments performed with MSO it appears that the GS-GOGAT pathway is responsible for photorespiratory NH_3 reassimilation, in spite of the presence of GDH in the mitochondrion (Wallsgrove et al., 1983). The original photorespiratory N cycle proposed that NH_3 reassimilation was via cytoplasmic GS. From C_2 studies on spinach chloroplasts it was suggested that chloroplastic GS is responsible for reassimilation. In spite of this the cytoplasm contains high levels of ATP in the light (Stitt et al., 1982) and may thus be a suitable location for reassimilation (Wallsgrove et al., 1983). Recent evidence from studies using mutants of barley with lesions in the photorespiratory pathway (absence of ferredoxin-dependent glutamate synthase and the inability to transport dicarboxylate substrates into the chloroplast) suggest that chloroplastic ferredoxin-dependent glutamate synthase activity may be mainly responsible for NH_3 reassimilation (Kendall et al., 1986; Wallsgrove et al., 1986).

Several studies show that the addition of MSO to leaves in the light under photorespiratory and non-photorespiratory conditions leads to an accumulation of NH_3 . MSO induced NH_3 accumulation has been used to provide a minimum estimate of the rate of photorespiration. From such studies on wheat and maize minimal rates of photorespiration were calculated to be 1-4% of CO_2 assimilation (Berger and Fock, 1983; 1985). Using [^{15}N]glycine incorporation as an indicator of photorespiration much higher rates (24% CO_2 assimilation) were determined (Berger and Fock, 1985). Accumulation of NH_3 stimulated by MSO supply has been suggested to uncouple photophosphorylation and thus inhibit CO_2 assimilation. The inhibitory effect of MSO on carbon assimilation has however been recently attributed to depletion of Calvin cycle intermediates rather than an uncoupling effect (Ikeda et al., 1984).

The possibility that NO_3^- reduction could be linked to photorespiration for the provision of reductant was raised by

Lips (1971). Photorespiration was proposed to function in two capacities:

- a fast efficient regulatory system coordinating nitrogen and carbon metabolism
- as source of reducing equivalents coupling NO_3^- reduction to the pentose phosphate (Lips, 1977).

This author suggested that NADH for NO_3^- reduction could be derived from the metabolism of glycolate to glyoxylate. Glycolate was found to stimulate the induction of NR which was suggested to be located in the same organelle as the enzyme (glycolate dehydrogenase) for photorespiratory glycolate metabolism (Lips, 1971). Roth-Bejerano (1973) reported that glycolate dehydrogenase was only found in barley and tobacco plants in the presence of NR and that plants supplied with adequate NO_3^- and NR were able to reduce NAD with glycolate. In contrast Mann et al (1978) have reported that glycolate was ineffective as a source of reductant for NR. Kaplan and Lips (1984) have taken up the proposals of Lips (1971) and produced a more detailed analysis. These authors found identical changes in NR and GDH activities following changes in NO_3^- and molybdenum nutrition.

8. METHODS AND MATERIALS

8.1. PLANT MATERIAL

8.1.1. Culture of *Lycopersicon esculentum*

Seeds of *Lycopersicon esculentum* var. Heinz (supplied by Straathof's Seeds, Johannesburg) were germinated in river sand at a density of approximately 0.5 cm^{-2} . Long Ashton nutrient medium (Hewitt, 1952) containing 14.8 mM NO_3^- (Table 1) was supplied (unless otherwise specified) twice weekly and tap water was supplied on other days. Where plants were starved of nitrogen, the Long Ashton nutrient medium was supplied as normal except that the KNO_3 and $\text{Ca}(\text{NO}_3)_2$ were omitted. After release from N starvation by provision of the complete Long Ashton medium, this N free treatment was continued until measurements were taken. The plants were grown in a phytotron chamber with a day/night temperature of $25/20^\circ\text{C}$ and day/night relative humidity of 70/60 %. The photoperiod was 14 hours starting at 6 am with a light intensity of $100 \mu\text{E.m}^{-2}.\text{s}^{-1}$ (For spectral composition see Fig. 4a). Plants were used when they were 20-30 days old.

8.1.2. Culture of *Pisum sativum*

Pisum sativum var. Meteor (Swann Technol. Ltd., Herts, England) seeds were soaked in tap water for 24 hours with constant aeration before planting in vermiculite at a density of approximately 1 cm^{-2} . Long Ashton nutrient medium (Hewitt, 1952) containing 14.8 mM NO_3^- (Table 1) was supplied (unless otherwise specified) twice weekly and tap water was supplied on other days. Where plants were starved of nitrogen, the Long Ashton nutrient medium was supplied as normal except that the KNO_3 and $\text{Ca}(\text{NO}_3)_2$

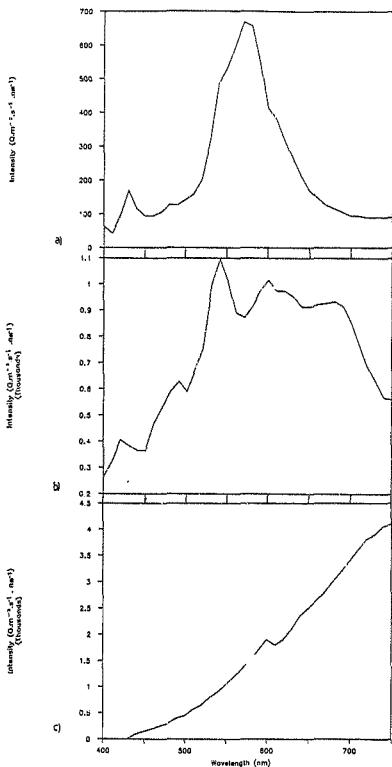


Fig. 4. Spectral analysis of light sources used for: a) Growth of plants; b) Gas exchange studies; c) Oxygen evolution studies.

were omitted. After release from N starvation by provision of the complete Long Ashton medium, this N free treatment was continued until measurements were taken. The plants were grown in a phytotron chamber with a day/night temperature of 20/15°C and day/night Relative Humidity of 70/60 %. The photoperiod was 14 hours starting at 6 am with a light intensity of $100 \mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ (For spectral composition see Fig. 4a). For plants used for protoplast extraction the photoperiod was started at 10 am. The photoperiod was set to begin late in the day so as to allow harvesting of tissue in dark conditioned plants. Tissue was collected from 18-25 day old plants.

Table 1. Long Ashton Nutrient Medium

Compound	Concentration ($\text{g}\cdot\text{l}^{-1}$)
KNO_3	0.505
$\text{Ca}(\text{NO}_3)_2$	0.820
FeEDTA	0.030
<u>Micronutrients</u>	
$\text{NaH}_2\text{PO}_4\cdot 2\text{H}_2\text{O}$	0.208
$\text{MgSO}_4\cdot 7\text{H}_2\text{O}$	0.369
MnSO_4	0.224×10^{-2}
$\text{CuSO}_4\cdot 5\text{H}_2\text{O}$	0.024×10^{-2}
$\text{ZnSO}_4\cdot 7\text{H}_2\text{O}$	0.029×10^{-2}
H_3BO_3	0.186×10^{-2}
$(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}\cdot 4\text{H}_2\text{O}$	0.004×10^{-2}
$\text{CoSO}_4\cdot 7\text{H}_2\text{O}$	0.003×10^{-2}
NaCl	0.585×10^{-2}

8.2. CO₂ GAS EXCHANGE ANALYSIS

8.2.1. Photosynthetic CO₂ fixation

Gas exchange measurements were carried out by means of an

Analytical Development Company Series 225 infrared gas analyzer (I.R.G.A.) in an open "leak proof" system. That the system was "leak proof" was established by closing the system and depleting the CO_2 levels with soda lime and then monitoring the change in CO_2 levels in the system with respect to time. The leak rate was found to be $< 1 \text{ ppm.h}^{-1}$ which was insignificant in relation to the rate of uptake of CO_2 by the plant in an open system and the sensitivity of the machine. The I.R.G.A. was set to read on differential (continuous monitoring of a reference air stream and analysis stream) for improved sensitivity (sensitivity $\pm 0.1 \text{ ppm}$). The incoming air was drawn from a mast on the roof and buffered in two 50 l drums before being dehumidified by a coil trap (Fig. 5). The cuvette used had a volume of 1.2 l and was large enough to allow a pea shoot to be stood in a small beaker of water inside the cuvette. The cuvette was surrounded on the sides and bottom by a water jacket and a flat glass plate was used to seal the top. A thermocouple was inserted into the cuvette to monitor cuvette temperature. A multiple entry system was used for the air inlet and the air in the cuvette was stirred by a fan driven by a magnetic stirrer. The fan was shown to be adequate in mixing the air through the observation that smoke introduced through the inlet of the cuvette mixed immediately. A port was provided through the wall of the water jacket to allow injection of solutions into the bathing medium of the shoot without opening the cuvette.

A manometer was used to ensure that pressure in the cuvette remained at the atmospheric level. This was achieved by using a non return bleed off valve. Relative humidity was measured using a Licor RH meter. Both the gas from the cuvette and that from the reference line were dried with silica gel and passed through filters to remove particulate matter before being passed into the I.R.G.A.. The flow rate of gas through both the cuvette and the reference line was maintained at 1 l.min^{-1} .

Light provided by a 400 W Siemens mercury vapour lamp was passed through a shallow flat sided perspex cooled water bath to remove the infrared component of the light. The light intensity delivered by this set up was determined as $500 \mu\text{E.m}^{-2}.\text{s}^{-1}$ and the

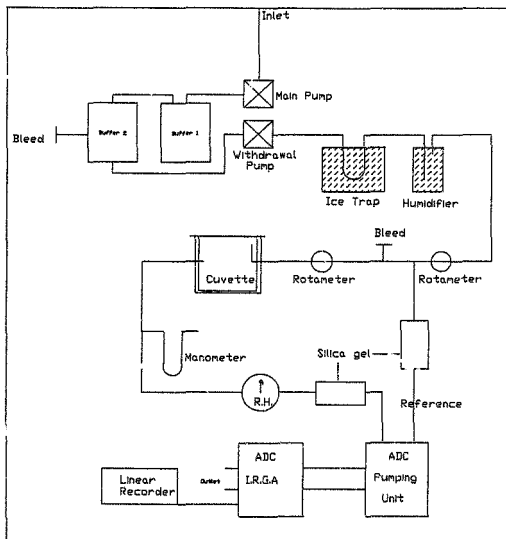


Fig. 5. The gas circuit employed in the determination of photosynthetic CO_2 uptake using the ADC I.R.G.A.

spectral composition (Fig. 4b) determined using a Tectum Instruments Quantaspectrophotometer (QSM 2500). The cuvette was protected from diffuse room light by surrounding the cuvette with a screen. Light intensity was varied using layers of shade cloth (layers of black nylon 4 mm mesh) placed over the cuvette.

Roots were removed from the shoots under water and the shoots transferred to a small beaker without exposing the cut surface to air. The beaker was closed with a suitably cut rubber stopper which served to reduce evaporation from the water surface and to support the shoot. Two teflon capillary tubes were inserted through the stopper to allow replacement of the bathing solutions. The exchange of the solutions was accomplished by using a two compartment container connected to a peristaltic pump. Pumping of the solution into the beaker from the container resulted in the bathing medium in the beaker being sucked back into one of the compartments of the container. Thus controlled exchange of bathing media could be accomplished without opening the cuvette and with little perturbation of the system. The bathing media were only exchanged once the CO_2 uptake had stabilized and remained constant for at least 10 minutes. The detached shoots were exposed to varying concentrations of NaNO_3 , NaNO_2 and NH_4SO_4 .

Low oxygen tensions were achieved by replacing the normal gas supply prior to the ice trap (Fig. 5) with a supply of 340 ppm CO_2 mixed from cylinder N_2 (5 ppm O_2) and cylinder CO_2 (1100 ppm CO_2 and <2 ppm O_2) with Wösthuff precision gas mixing pumps.

Photosynthetic rates were calculated from ppm CO_2 taken up and expressed either on the basis of leaf area (determined using a Licor planimeter) or chlorophyll concentration.

Chlorophyll concentration was determined according to the method of Arnon (1949). Leaf material (0.1 g) was thoroughly homogenized in a mortar and pestle with acid washed sand in 20 ml 80% acetone, filtered through Whatman No.1 filter paper and the resulting filtrate read on a Varian DMS 90 spectrophotometer at 645 and 663 nm. The amount of chlorophyll was calculated as fol-

lows (Wintermans and De Mots, 1965):

Chlorophyll (mg.g⁻¹ fresh weight) =

$$(20.2 \times \text{Abs}_{645}) + (8.02 \times \text{Abs}_{663}) \times V / (1000 \times W)$$

where: Abs_{645} = absorption at 645 nm
 Abs_{663} = absorption at 663 nm
V = Volume of acetone used (ml)
W = Amount tissue used (g)
1/1000 = Conversion of chlorophyll concentration
from g.l⁻¹ to mg.ml⁻¹.

The photosynthetic rate was calculated as follows:

Photosynthetic rate (mg CO₂.dm⁻².h⁻¹).

OR

$$(\text{mg CO}_2.\text{mg chl}^{-1}.\text{h}^{-1}) =$$

$$(\Delta \text{ppm} / \text{LA}) \times ((\text{FR} \times 1000) / 60) \times (44 / 22.414) \times (273 / T) \times (P / 1013) \times 3.6$$

Where: Δppm = CO₂ uptake (ppm)
LA = Leaf area (dm²) or chlorophyll
(mg chl⁻¹.g fwt⁻¹)
FR = Flow rate (l.min⁻¹)
T = Temperature (K)
P = Pressure (mBar)

Calculation of photosynthetic rates was performed using a program written for the IBM PC in BASICA (Appendix A).

8.2.2. Carbon dioxide compensation points

Compensation points (CCP) were determined using an Analytical Development Company Series 225 infrared gas analyzer coupled into a closed gas circuit. The system was checked for leaks by circulating CO₂ depleted air through the system and the leak rate

was determined to be $0.4 \text{ ppm} \cdot \text{h}^{-1}$ which was small in comparison to the potential of the plants used for uptake. The I.R.G.A was set to read on absolute with a flow rate of $1 \text{ l} \cdot \text{min}^{-1}$. Before determination of the CCP the plants were allowed to photosynthesize in an open system until a stable rate of photosynthetic CO_2 fixation was observed whereupon the system was closed using a valve. Initial air supply to the open system was drawn from a mast on the roof through two 50 l drums and pressure in the system equalized using a non-return bleed off valve. The cuvette used was of a dome shaped construction sealing onto a glass plate and had a volume of 1 l. A multiple entry system was used for the air inlet and the air was stirred using a fan driven by a magnetic stirrer. The plants were stood in a beaker inside the cuvette and a port was provided in the side of the cuvette to allow the introduction of salts to the bathing medium of the shoots. Light was provided by a 400 W Siemens mercury vapour lamp at an intensity of $600 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ (For spectral composition see Fig. 4b). The cuvette had no water jacket but the temperature was maintained between $21\text{--}25^\circ\text{C}$ (as determined using a thermocouple) with a desk fan. The air passing into the I.R.G.A was dried using a silica gel column.

Conditions of low oxygen tensions were achieved by flushing the gas system with a supply of 340 ppm CO_2 mixed from cylinder N_2 (5 ppm O_2) and cylinder CO_2 (1100 ppm CO_2 and <2 ppm O_2) with Wösthuff precision gas mixing pumps.

Roots were removed from the shoots under water and the shoots transferred to a small beaker without exposing the cut surface to air. The beaker was closed with a suitably cut rubber stopper which served to reduce evaporation from the water surface and to support the shoot. Two teflon capillary tubes were inserted through the stopper to allow replacement of the bathing solutions through the entry port provided. The bathing medium was exchanged after observation of a stable CCP by repeatedly removing and replacing partial volumes in the beaker with a syringe. The detached shoots were exposed to varying concentrations of NaNO_3 , NaNO_2 and NH_4SO_4 . The CCP was expressed in ppm.

8.3. STOMATAL RESISTANCE

The stomatal resistance of leaves of pea grown on normal strength Long Ashton medium was measured using a Licor Autoporometer (automatic diffusive resistance meter) with an Li 20S sensor set to read between 20% and 40% R H. The instrument was calibrated with the calibration plate provided according to the operating instructions for this instrument and under the same conditions under which measurement of leaf stomatal resistances was performed (26°C and 600 $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$). The calibration curve was plotted using a 'best-fit' procedure and the resistances calculated from the equation for this line.

The stomatal resistances of normal rooted peas and peas with the roots removed were determined. The roots were removed by cutting under water, and the shoots were then transferred to vials, without exposure of the cut surface to air. The changes in the stomatal resistance due to cutting and the subsequent recovery were followed over time. In addition the response of cut shoots to transfer into a bathing medium containing 10 or 20 mM NaNO_3 or NH_4Cl was followed over time.

8.4. LEAF DISK CARBON DIOXIDE FIXATION

Leaf disks (1 cm diameter) were punched from leaves of pea grown with a supply of normal strength Long Ashton medium twice weekly as described in section 8.1.2. During the punching operation the leaves were stored on wet filter paper. The disks were then transferred to mesh bags which were immersed in water. The bags were placed in beakers containing different nitrogen salts and vacuum infiltrated for two cycles of 45 seconds. The bags were then rinsed in distilled water before the leaves were placed dorsal surface up onto wet filter discs. The labeling of the discs was accomplished in a specially designed cuvette which held six filter discs. The cuvette consisted of a six wells bored into 4 cm thick perspex. Each well was supplied with a gas inlet-outlet, a control valve and a flat perspex cover. The cover had a baffle positioned to mix the gas being input to the cuvette and the

cover was sealed onto the cuvette using vacuum grease. The gas was drawn through the cuvette by a pump at $1 \text{ l} \cdot \text{min}^{-1}$ from a cylinder containing $^{14}\text{CO}_2$ connected to a buffering tank and a bleed off valve. The exhaust gas was passed through a "Carbosorb" soda lime column.

After labeling the discs they were transferred to scintillation vials and immediately immersed in 5 ml boiling 80% ethanol and the vials capped. After cooling the leaf disks were ground in the ethanol solution with a mechanical glass-glass homogenizer. To the homogenate was added 1 ml 20% (w/v) TCA (to release any unfixed $^{14}\text{CO}_2$) and 1 ml 10 mM phenylhydrazine (to prevent volatilization of organic acids). The vials were stood uncapped in a fume hood for 30 minutes before being made up to 10 ml with water. A 0.2 ml aliquot of sample was added to 10 ml "Aqualgel" scintillation fluid supplied by Packard. The samples were counted on a Packard liquid scintillation counter. The instrument was set to provide disintegrations per minute using the channels ratio technique to correct for quenching. The photosynthetic assimilation of $^{14}\text{CO}_2$ was expressed on the basis of the leaf disc area as $\text{dpm} \cdot \text{m}^2 \cdot \text{s}^{-1}$.

8.5. PROTOPLAST ISOLATION

A modification of the methods described by Leegood et al. (1982) was used. The leaves were taken from plants grown with a supply of normal strength Long Ashton medium twice weekly and exposed to 14 h dark. About 3 g leaf material was either cut with a sharp blade into 0.5 - 1 mm strips or lightly rubbed with carborundum powder to score the epidermis. The leaves were then washed for about 15 minutes in 0.5 M sorbitol to remove ruptured cell contents and carborundum. The leaf material was then incubated in 20 ml 2.5 % Cellulase "Onozuka R10" (Yakult Biochemicals Co., Japan) or 2 % Cellulase "Onozuka RS" (Yakult Biochemicals Co., Japan), 0.25 % Macerozyme "Onozuka R10" (Yakult Biochemicals Co., Japan), 0.5 % Hemicellulase (Sigma), 0.5 M sorbitol, 1 mM CaCl_2 , 5 mM MES (pH 5.5), 0.05 % BSA (pH 5.5) at 25°C in a 9 cm diameter petri dish with constant shaking on a IKA-WERK (KS 500) shaking table

at about 70 cycles.min⁻¹ for 2½ - 3 hours. The digesting medium was passed through a 500 µm mesh and a 88 µm Nybolt mesh. The leaf material was washed three times with 10 ml chilled 0.5 M sorbitol, 1 mM CaCl₂, 5 mM MES (pH 6.0) and similarly filtered. The filtrate was spun in a Hettich Universal table top swing out centrifuge at 100 g for 5 min. The supernatant was discarded and the pellet resuspended in chilled 0.5 M sorbitol, 1 mM CaCl₂, 5 mM MES (pH 6.0). The protoplast suspension was kept on ice in the light on a shaking table (90 cycles.min⁻¹).

The protoplasts were counted on a haemocytometer using Evans Blue vital dye (0.2 g/100 ml) in 0.7 M sorbitol to determine intactness. The protoplasts were discarded if intactness fell below 60 %. Protoplast counts (intact) were found to be high (5.04×10^6 .ml⁻¹ ± S.D. 0.71×10^6 .ml⁻¹) and intactness varied with the preparation but was characteristically over 90%.

All measurements using protoplasts were expressed on the basis of chlorophyll. Chlorophyll concentration was estimated by determining the absorbance of 50 µl of protoplast suspension in 20 ml 80 % acetone (v/v) at 663 nm on a Varian DMS 90. The absorbance was substituted into the following formula (modified from Arnon, 1949):

$$\text{Vol. } (\mu\text{l}) = 9/\text{Abs}_{663}$$

where:

Abs₆₆₃ = Absorbance at 663 nm
Vol. = Volume (µl) of protoplast suspension required for 100 µg chlorophyll.

8.6. PROTOPLAST NITRATE, NITRITE AND AMMONIUM NET UPTAKE STUDIES

The protoplasts were incubated in buffer containing 0.4 M sorbitol, 10 mM NaHCO₃, 0.5 mM CaCl₂, 0.1 mM KH₂PO₄ and 50 mM HEPES buffer (pH 7.6) at about 20°C under a light intensity of 600 µE.m⁻².s⁻¹ using a sufficient volume of protoplasts to make up a solution containing 300 µg chl.ml⁻¹. Nitrate, nitrite and

ammonium were added as NaNO_3 , NaNO_2 , NH_4SO_4 . Each concentration was replicated three times and a control with no added nitrogen was included. Before addition of the nitrogen salts the protoplasts were pre-incubated for 10 minutes to ensure a fully induced photosynthetic system. The protoplast suspension was shaken on a shaking table at $100 \text{ cycles} \cdot \text{min}^{-1}$ in small vials (3 ml) with a glass bead to retain the protoplasts in suspension. The vials were sealed with a thin plastic film to prevent evaporation. At various time intervals aliquots of the protoplasts suspension were removed and immediately centrifuged at 8000 g for 60 s in a Beckman Microfuge II. The supernatant was then used for the determination of nitrate, nitrite and ammonia.

In order to replicate the experiments of Rathnam (1978) the assay medium used above was replaced with 0.4 M sorbitol, 0.75 mM MgCl_2 , 0.1 mM KH_2PO_4 , 50 mM Tricine-NaOH (pH 8.1) and 3 mM Na-isoascorbate. In addition the centrifugation step was omitted in some cases but it was found that the variability of the results was significantly increased under these circumstances.

8.6.1. Nitrate

8.6.1.1. Nitrate net uptake

Nitrate net uptake was measured using the nitration of salicylic acid. Using a modification of the Cataldo method (Cataldo et al., 1975) a $100 \text{ } \mu\text{l}$ aliquot of protoplasts was mixed with $400 \text{ } \mu\text{l}$ 1% (w/v) salicylate in concentrated H_2SO_4 . The mixture was allowed to stand for 20 minutes before the addition of 9.5 ml 2 M NaOH whereafter the samples were allowed to cool for 60 minutes. The samples were then read on a Varian DMS 90 spectrophotometer at 410 nm against a reagent blank and the concentrations of nitrate calculated using a standard curve similarly prepared, but using different nitrate concentrations in place of the protoplast suspension.

Experiments performed using this technique yielded no results be-

cause no change in the levels of NO_3 could be found. This may be attributable to the relative insensitivity of this method. In order to gain some idea of the activity of NR two techniques were employed to measure the in vitro activity of this enzyme.

8.6.1.1. Dark anaerobic assay for NR

Protoplasts isolated as above were added to phosphate buffer (pH 7.6) with 0.1 ml of the various concentrations of nitrate to make up a final volume of 1 ml. The protoplasts were then incubated in a water bath at 26°C in the dark in scintillation vials. The vials were sealed with suba-seal tops through which syringe needles were pushed to allow the passage of N_2 over the contents of the vial. The gas was not bubbled through the protoplast suspension in order to avoid breakage of the protoplasts. After a 30 minute incubation 0.3 ml 1.3 M zinc acetate was added followed by 0.7 ml phosphate buffer (pH 7.6) and 2 ml chloroform after which the preparation was centrifuged at 1500g in a Beckman TJ6 swing-out bucket centrifuge for 5 minutes. Supernatant (1 ml) was then removed for assay of nitrite using the method described below.

8.6.1.2. Benzyl viologen assay for NR

This assay was adapted from the description of an assay for NR in algae by Larsson et al. (1985b). A 0.5 ml volume of protoplasts was solubilized by vigorous shaking with 0.2 ml toluene for 1 minute. To this 0.5 ml of a solution containing 10 mM KNO_3 , 2mM benzyl viologen and 100 mM MES at pH 7.5 was added. The reaction was started with the addition of 1 mg $\text{Na}_2\text{S}_2\text{O}_4$ and terminated after 15 minutes by vigorous shaking in air. Zinc acetate (1 ml) and 2 ml chloroform were added prior to centrifugation at 1500g in a Beckman TJ6 swing-out bucket centrifuge for 5 minutes. Supernatant (1 ml) was then removed for assay of nitrite using the method described below.

8.6.2. Nitrite net uptake

Nitrite concentration was determined through the formation of a azo compound by the reaction of sulphanilamide in acidic solution with nitrite and thereafter N-(1-naphthyl)ethyleneamine dihydrochloride (Goltermann et al., 1978). For determination of nitrite 100 μ l aliquots of supernatant were added to 1.0 ml sulphanilamide solution (1 % (w/v) in 2 N HCl). To this mixture 1.0 ml of N-(1-naphthyl)ethylenediamine dihydrochloride (0.02 % (w/v) in distilled water) was added. The colour was allowed to develop for 30 minutes and the absorbance was read on a Varian DMS 90 spectrophotometer at 540 nm against a reagent blank and the concentrations of nitrite calculated using a standard curve similarly prepared, but using different nitrite concentrations in place of the protoplast suspension.

8.6.3. Ammonium net uptake

The phenyl-hypochlorite method (Scheiner, 1975) was used for determining ammonium nitrogen concentration. Indophenol blue is produced by reacting phenol with alkaline hypochlorite and the blue colour may be enhanced with sodium nitroprusside. The procedure includes the use of EDTA (Ethylene-dinitrilo tetraacetic acid) for suppression of interference from copper, hydroxides and phosphates. An alkaline buffer is also included to reduce the effects of variations in sample acidity, although this was not expected to be a problem with the protoplast supernatant which was buffered.

A 100 μ l aliquot of supernatant from the protoplast suspension was added to 8 ml 0.12 % di-sodium EDTA. To this was added 1 ml phenolic nitroprusside which was prepared daily from equal parts of sodium nitroprusside (0.5 g sodium nitroprusside in 100 ml distilled water) and ethanolic phenol (10 g phenol in 100 ml 95 % ethanol). Immediately thereafter 1 ml buffered alkaline phosphate prepared daily from 4 parts alkaline phosphate buffer (26.84 g $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ and 20.65 g NaOH in 1 l distilled water) and 1 part 1.5 % sodium hypochlorite (diluted daily) was added. The colour

was allowed to develop for 60 minutes before the absorbance was measured at 635 nm on a Varian DMS 90 against a reagent blank and the concentrations of ammonium circulated using a standard curve similarly prepared, but using different ammonium concentrations in place of the protoplast suspension.

8.7. PROTOPLAST CARBON DIOXIDE FIXATION

Carbon dioxide fixation was measured in buffer containing 0.4 M sorbitol, 6 mM NaHCO_3 , 5 mM CaCl_2 and 50 mM HEPES buffer (pH 7.6) (as described by Leegood et al., 1982) at 20°C under a light intensity of $600 \mu\text{E.m}^{-2}.\text{s}^{-1}$ or $100 \mu\text{E.m}^{-2}.\text{s}^{-1}$ using a sufficient volume of protoplasts to make up a solution containing $100 \mu\text{g chl.ml}^{-1}$. The experiments were conducted in scintillation vials continuously stirred at low speed with magnetic stirrers to prevent sedimentation of the protoplasts. Carbon assimilation was determined using 10 mM $\text{NaH}^{14}\text{CO}_3$ (0.01 mCi.ml^{-1}) added to the above buffer. At various times 100 μl aliquots of labeled sample were transferred to scintillation vials containing 100 μl 20 % (w/v) trichloroacetic acid to kill protoplasts and release unfixed $^{14}\text{CO}_2$ and 100 μl 10 mM phenylhydrazine to stabilize volatile organic acids. The samples were allowed to stand for 30 minutes in a fume hood to allow the escape of any unfixed $^{14}\text{CO}_2$.

A 10 ml volume of scintillation fluid, consisting of 70 % (v/v) toluene, 30 % (v/v) ethanol, 6 g.l^{-1} 2-5 diphenyl oxazole (PPO) as a primary scintillator and 300 mg.l^{-1} 1,4-di(2-(5-phenyloxazolyl))-benzene (POPOP) as a secondary scintillator, was mixed with the sample. This was followed by counting for 10 minutes per sample. With each set of samples blanks containing all reagents but no protoplasts were included. Each concentration was replicated three times and sample blanks were included to which no nitrogen was added. The samples were counted on a Packard liquid scintillation counter. The instrument was set to provide disintegrations per minute using the channels ratio technique to correct for quenching.

8.8. PROTOPLAST OXYGEN EVOLUTION

Oxygen evolution by protoplasts was followed polarographically using a Clark-type Rank electrode coupled to a MSE Spectroplus spectrophotometer (for signal detection) and a Linear chart recorder. The platinum-silver electrode was covered with a 1 M KCl solution under a square of lens tissue to ensure even distribution of the KCl and the whole assembly isolated from the sample chamber with a teflon membrane. The water cooled oxygen electrode was positioned in front of a 150 W tungsten lamp capable of delivering in excess of $600 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ to the sample chamber (for spectral composition see Fig. 4c). The oxygen electrode was calibrated using O_2 saturated aerated water taken to contain $0.28 \mu\text{moles } \text{O}_2 \cdot \text{ml}^{-1}$ at 20°C and sodium dithionite to deplete all O_2 from solution. Protoplast suspension (0.2 ml), pre-incubated in dark unless otherwise specified, was added to 2.8 ml of assay medium consisting of 0.4 M sorbitol, 0.75 mM CaCl_2 and 50 mM HEPES (pH 7.6) (as described by Leegood et al., 1982) at 20°C stirred at $100 \text{ cycles} \cdot \text{min}^{-1}$. In addition 10 mM NaHCO_3 was included in the medium unless otherwise stated. For experiments in which different concentrations of NaHCO_3 were used, the CO_2 in the medium was removed by deaeration under vacuum followed by the bubbling of CO_2 free air (passed through a soda lime column) through the medium prior to use and constantly while in use. The effect of the addition of various nitrogen salts was determined as the change in rate of O_2 evolution from a constant rate determined over about 5 min. The rate of oxygen evolution was expressed as $\mu\text{moles } \text{O}_2 \cdot \text{mg chl}^{-1} \cdot \text{h}^{-1}$. The results were subjected to a homogeneity of slopes test using SAS and only highly significant results were considered.

Nitrate, nitrite, ammonia and phosphate were added as NaNO_3 , NaNO_2 , NH_4SO_4 and Na_3PO_4 respectively. Sodium chloride was used as a control to ascertain the osmotic effects of salts added to the buffer.

8.9. PROTOPLAST ATP DETERMINATION

ATP determinations were made using the luciferin/luciferase system (Weil Organization) and an LKB luminometer with an integrator and printing unit. The protoplasts were incubated in buffer containing 0.4 M sorbitol, 10 mM NaHCO_3 , 0.5 mM CaCl_2 and 50 mM HEPES buffer (pH 7.6) at 20°C under a light intensity of 600 $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ using a sufficient volume of protoplasts to make up a solution containing 300 $\mu\text{g chl}\cdot\text{ml}^{-1}$. The protoplast suspension was stirred in scintillation vials with magnetic stirrers at low speed to retain the protoplasts in suspension. Aliquots (150 μl) were transferred to tubes cooled in liquid nitrogen. Just before the measurement of the ATP levels, the samples were thawed (about 30 s), 100 μl suspension was mixed with 100 μl of somatic releasing agent (Weil Organization), the mixture shaken for 30 s and the tube inserted into the luminometer. Thereafter 100 μl of luciferin/luciferase suspended in HEPES buffer at pH 7.5 was injected using a micro syringe and 10 s after the start of the incubation, integration of 10 s counts was initiated. The concentrations of ATP were calculated using a standard curve.

9. RESULTS

9.1. SHOOT PHOTOSYNTHETIC CARBON ASSIMILATION

9.1.1. *Lycopersicon esculentum*

In initial experiments it was established that the supply of inorganic nitrogen in the nutrient medium used for the culture of the plants had important consequences for the response of the plants to various treatments. The effects of withholding nitrogen from the culture medium for varying periods was examined. These effects may be termed 'long term' involving the elapse of between 8 hours and 4 days whereas the primary aim of this investigation was to determine the 'short term' effects of inorganic nitrogen. Short term effects were determined by monitoring photosynthetic and other parameters during and immediately after the addition of inorganic nitrogen. Unless otherwise stated all responses to supply of inorganic nitrogen are considered "short term".

9.1.1.1. Pre-treatment effects

Withholding nitrogen resulted in chlorotic plants with stunted shoots but very extensive rooting systems. Photosynthetic rates of these shoots were found to be significantly lower than those of plants exposed to normal nitrogen nutrition (Fig. 6). Supply of normal Long Ashton medium to plants initially starved of nitrogen resulted in an increase in net photosynthetic rate in all cases. The magnitude of this increase was dependent on when the photosynthetic rate was determined after supply. The effect of the nitrogen appeared to be relatively slow in becoming manifest with a maximum effect occurring only after 3 days. The

supply of nitrogen to initially starved plants resulted in a greater photosynthetic rate after 2 days than that observed for plants fed nitrogen on a regular basis (Fig. 6).

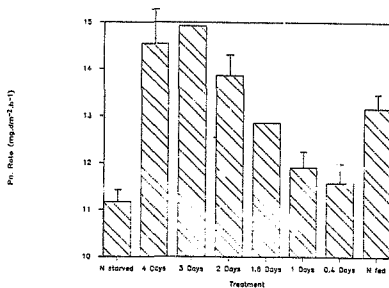


Fig. 6. Comparison of effects of release from N starvation at variable times prior to determination of the photosynthetic rates of *L. esculentum*. Bars indicate S.E. where sufficient measurements were performed to calculate this statistic.

The pre-treatment was also found to have some effects on the response of the plants to nitrogen introduced into the bathing medium of shoots from which the roots had been removed. Plants starved of nitrogen showed no short term response to 1 mM NaNO_3 (Fig. 7). Plants given nitrogen for the first time 8 hours before the photosynthetic measurement also showed no net response. The largest short term response to nitrogen was observed 1.6 days after treatment (Fig. 7). These results are expressed as a percentage change from a basal rate. This was necessary to facilitate comparison between plants which did not show the same initial photosynthetic rates. The basal rate was taken from the phase before addition of the nitrogen in which no change in rate was observed for at least 10 minutes.

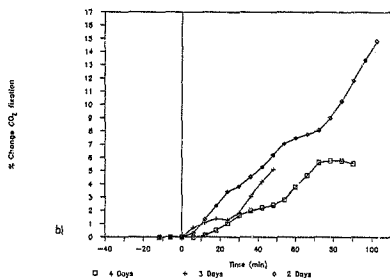
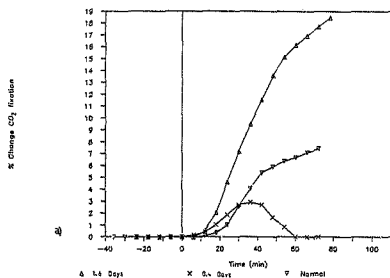


Fig. 7. The response of *L. esculentum* to 1 mM NaNO_3 supplied to plants released from N starvation at various times prior to determination of photosynthetic rates. a) N supplied regularly (Normal), 0.4 and 1.6 days prior to measurement b) N supplied 2, 3 and 4 days prior to measurement. S.E. < 1.2.

9.1.1.2. The influence of nitrate and ammonium

The response to the supply of nitrate was followed over a period of up to 124 minutes from the time of exposure to nitrate. Variability presented a problem with studies using the I.R.G.A. particularly as a result of the destructive nature of the technique. This held true for absolute rates as well as responses to inorganic nitrogen. The reduction of light intensity from $600 \mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ to $200 \mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ had no effect on the responses observed other than to reduce the initial photosynthetic rate. The response of the plants to nitrate supply in the concentration range 0.1 mM to 10 mM is shown in Fig. 8. It is apparent from these curves that there was no lag between addition of the salt and the response. The effect was also markedly concentration dependent with both stimulation and inhibition being observed. Concentrations below 1 mM were generally found to stimulate photosynthetic CO_2 fixation while 10 mM nitrate was inhibitory. The largest stimulation was found to result from supply of 0.2 mM nitrate (Fig. 9). With increasing concentration an initial stimulation followed by a subsequent inhibition of photosynthetic rate was observed.

Supply of ammonium had a stimulatory effect on photosynthetic CO_2 assimilation. Ammonium at 0.3 mM had greater stimulatory effect than did 1 mM ammonium (Fig. 10).

9.1.2. Pisum sativum

9.1.2.1. Pre-treatment effects

The effect of withholding nitrogen from peas was found to be completely different to the effect on tomato. The pea plants exhibited only slightly lighter green leaves. No stunting of growth was apparent and the roots appeared to be normally developed. A notable difference between the photosynthetic CO_2 fixation rates of pea (about $5 \text{ mg}\cdot\text{dm}^{-2}\cdot\text{s}^{-1}$) and tomato (about $13 \text{ mg}\cdot\text{dm}^{-2}\cdot\text{s}^{-1}$) was that the pea rates were lower than those of tomato

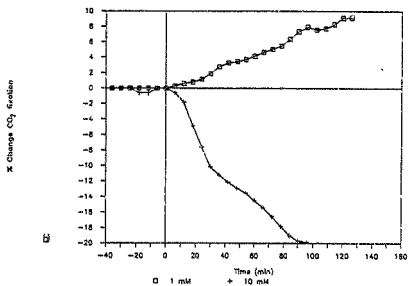
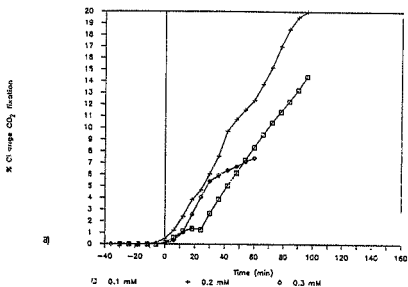


Fig. 8. The effect of feeding various concentrations of NO_3^- to the bathing medium of cut normal N fed shoots of *L. esculantum* on the rate of photosynthetic carbon assimilation. a) Supply in range 0.1 mM - 0.3 mM b) Supply in range 1 mM - 10 mM. S.E. < 2.4.

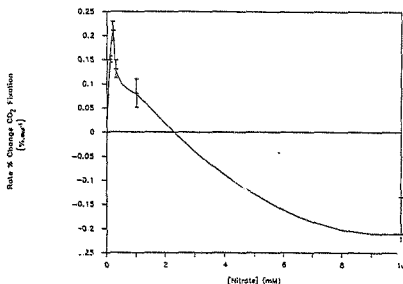


Fig. 9. Summary of the response of *L. esculentum* shoot photosynthetic rate to NO_3^- . Rate % change was derived from the slope of the linear best-fit line of % change over time. Although all data points were retained, use of splines was made to derive shape of curve.

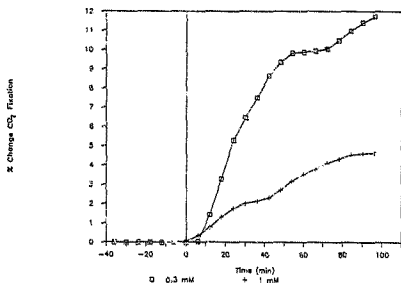


Fig. 10. The affect of feeding various concentrations (0.1 mM - 0.3 mM) of NH_4^+ to the bathing medium of cut shoots of *L. esculentum* on the rate of photosynthetic carbon assimilation. S.E. < 3.1.

(Fig. 6 and Fig. 11a). Plants starved of nitrogen exhibited only slightly ($<0.5 \text{ mg CO}_2 \cdot \text{dm}^{-2} \cdot \text{h}^{-1}$) reduced photosynthetic rates in comparison to plants supplied with nitrogen. The supply of Long Ashton medium containing nitrogen to previously starved plants brought about a stimulation of the photosynthetic rate within a few hours (Fig. 11a) and this rate was greater than that of plants normally supplied with nitrogen. When the data was expressed on the basis of chlorophyll rather than leaf area the patterns of response were quite distinct (Fig. 11b). In this case the nitrogen starved plants appeared to exhibit a higher photosynthetic rate than the normal nitrogen fed plants. This was probably the result of the slight chlorosis of the pea shoots. Again the supply of nitrogen containing Long Ashton medium a few hours prior to photosynthetic determination appeared to increase the photosynthetic rate.

9.1.2.2. The influence of nitrate and ammonium

Supply of varying concentrations of nitrate and ammonium to pea shoots had similar resultant response patterns to those of tomato. Sodium chloride supplied at 1 mM appeared to have no effect. Concentrations of nitrate in the range 0.3 mM - 10 mM had stimulatory effects on the rate of photosynthetic CO_2 assimilation (Fig. 12). The largest response was found with 1 mM NO_3^- . Supply of 25 mM NO_3^- had a strong inhibitory effect on CO_2 fixation. Pea seemed to be less sensitive to nitrate than the tomato with a maximum response of 5% as opposed to 20%. In the case of the pea there was a lag phase lasting 6-18 minutes before a response occurred.

Ammonium elicited only a very small stimulation of photosynthetic CO_2 assimilation at 0.3 mM, at higher concentrations (1 mM - 10 mM) ammonium had an inhibitory influence on CO_2 assimilation (Fig. 13). This pattern may be contrasted with that for tomato (Fig. 10) where 1 mM had a positive influence on photosynthetic CO_2 assimilation. Although no lag was apparent (Fig. 13) it is possible that this occurred in the 6 minute period after addition, before the first point of the curve was taken.

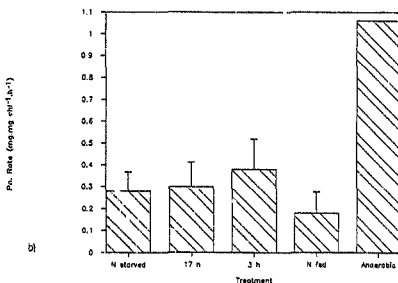
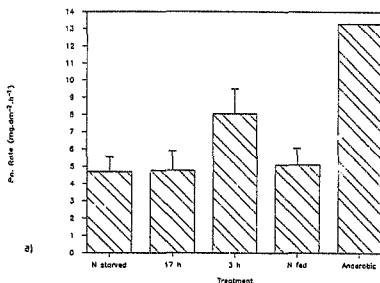


Fig. 11. Photosynthetic rates of *P. sativum* either N starved or released from starvation at various times prior to measurement as compared with normally N fed plants. Effect of anaerobic conditions on photosynthetic rates of normally N fed plants included to illustrate importance of CO₂ dissimilation in determining photosynthetic rates. Photosynthetic rates expressed: a) on basis of leaf area b) on basis of chlorophyll content. Bars indicate S.E. where sufficient measurements were performed to calculate this statistic.

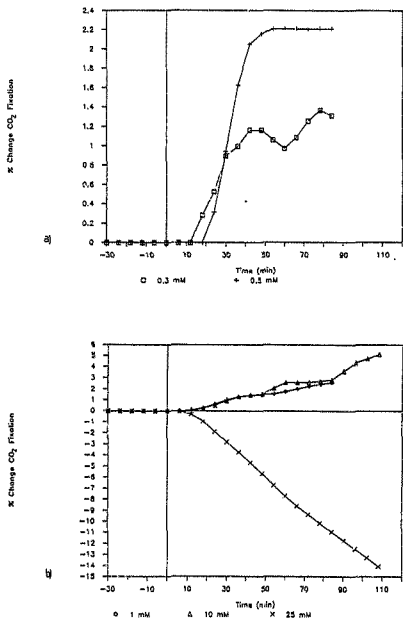


Fig. 12. The consequence of feeding various concentrations of NO_3^- to the bathing medium of cut shoots of *P. sativum* on the rate of photosynthetic carbon assimilation. a) Supply in range 0.3 mM - 0.5 mM. S.E. < 0.41. b) Supply in range 1 mM - 25 mM. S.E. < 1.1.

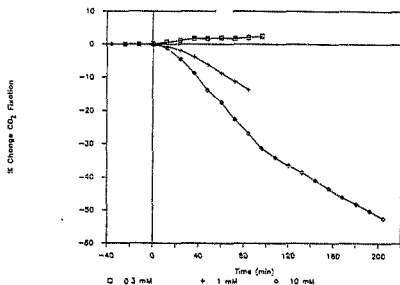


Fig. 13. The effect of feeding various concentrations (0.3 mM - 10 mM) of NH_4^+ to the bathing medium of cut shoots of *P. sativum* on the rate of photosynthetic carbon assimilation. S.E. < 1.3 for 0.3 mM and 1 mM NH_4^+ but S.E. < 15.6 for 10 mM.

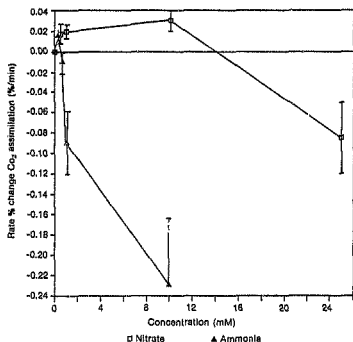


Fig. 14. Summary of the response of *P. sativum* shoot photosynthetic rate to NO_3^- and NH_4^+ . Rate % change was derived from the slope of the linear best-fit line of % change over time. Bars indicate S.E.

Scrutiny of Fig. 14 indicates that the concentration range over which nitrate was effective in stimulating CO_2 fixation was much broader than for tomato (Fig.9). Ammonia exhibited only a very transient enhancement before very rapidly becoming inhibitory.

9.1.2.3. Carbon dioxide compensation points

Compensation points were determined in order to ascertain whether the effect of inorganic nitrogen was related to net photosynthetic CO_2 fixation or to photorespiration. Although compensation points are arguably not the best method of determining photorespiration they are easily determined. Initial compensation points were found to depend strongly on the pre-treatment of the plants (Fig. 15).

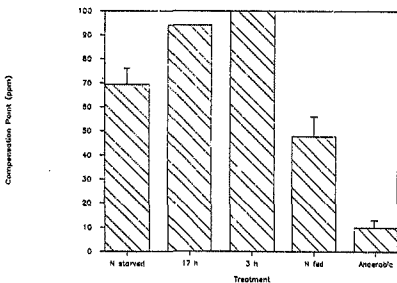


Fig. 15. Compensation points of *P. sativum* either N starved or released from starvation at various times prior to measurement as compared with normally N fed plants. Effect of anaerobic conditions on compensation points of normally N fed plants included to illustrate the dependence CO_2 dissimilation on the presence of O_2 . Bars indicate S.E. where sufficient measurements were performed to calculate this statistic.

Nitrogen starved plants were found to have significantly higher compensation points (69 ppm) than those of nitrogen fed plants (58 ppm). Supply of Long Ashton medium containing nitrogen resulted in higher compensation points at both 17 hours and 3 hours after supply. After 3 hours the compensation points (100 ppm) were greater than those determined after 17 hours (95 ppm).

Under low oxygen tension conditions normal nitrogen fed plants exhibited very low compensation points (10 ppm). This appeared to be correlated to enhanced gross photosynthetic CO_2 assimilation activity expressed both on the basis of leaf area and chlorophyll content (Fig. 11). Low oxygen tensions do not imply strictly anaerobic conditions. The gasses used to mix the CO_2 contained small quantities of O_2 and the use of a closed gas system would allow the accumulation of photosynthetically derived O_2 . When an attempt was made to measure the accumulation of O_2 in the closed gas system using a Hartmann and Braun paramagnetic O_2 detector however, no significant changes (not attributable to the measured leak rate) were detected within a 4 hour period although the instrument was sensitive to changes in O_2 concentration greater than 0.5% and the leak rate was 0.3 l.h^{-1} . It may be possible that the photosynthetic evolution of oxygen was small enough to be obscured by this leakage of O_2 into the gas system.

2.1.3. Limitation in the use of the I.R.G.A

One of the limitations of using intact shoots is that the supply of inorganic nitrogen to the shoot material is dependent on the shoot translocation system. This results in uncertain supply of the inorganic nitrogen to the photosynthetic tissues. In addition the removal of the roots can induce a trauma response (e.g. stomatal closure), although in all cases plants were allowed to equilibrate before experiments were conducted. A further limitation is in the replication of experiments. Only limited replication was possible as a result of the lengthy nature of the measurement of photosynthesis. As a result, replication from plants of the same age and at the same time of day was impossible.

To overcome these limitations, leaf discs and protoplasts were utilized. Although both systems are subject to criticisms as a result of their isolation from the plant, they provide the advantages of replicability, ease of manipulation and more rapid experimentation.

9.1.4. Stomatal Resistance

Removal of the roots from pea plants had an immediate effect on stomatal resistance. The resistance increased dramatically immediately after cutting and only recovered to levels similar to those prior to cutting after approximately 90 minutes (Fig. 16). The greatest increase occurred within the first 3 minutes but continued for up to 30 minutes after cutting whereafter the resistance values returned to approximately the values prior to cutting.

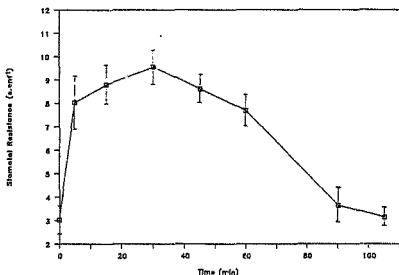


Fig. 16. The effect of excising the root from the pea plant on the stomatal resistance of the shoot. Bars indicate S.E.

Cut shoots allowed to equilibrate for 90 minutes after cutting were found to respond rapidly (<10 minutes) to transfer (without exposure of the cut surface to air) to bathing media containing various salts (NaCl, NaNO₃, NH₄Cl). These responses could however only be measured at fairly high salt concentrations (10 - 20 mM). At concentrations lower than these the effects, if any, were lost in the variability of the data. To allow comparison of the curves, the data were normalized around the mean initial value. Sodium chloride was used as a control salt to determine whether the effects were the result of the addition of the salt or the nitrogen salt specifically. Both 10 and 20 mM NaCl (Fig. 17a and Fig. 18a) increased the stomatal resistance above that of the control (kept in distilled water). In relation to the water control, NO₃⁻ appeared to have no effect, but in comparison with NaCl (Fig. 17b and Fig. 18b) nitrate caused a decreased stomatal resistance for both concentrations. Ammonium supply resulted in increased stomatal resistance in comparison to the water control at both 10 and 20 mM ammonium (Fig. 17a and Fig. 18a). In comparison with the effect of NaCl, 10 mM NH₄⁺ reduced stomatal resistance, but at the higher concentration, stomatal resistance was dramatically increased (Fig. 17b and Fig. 18b).

9.2. LEAF DISK PHOTOSYNTHETIC CARBON DIOXIDE ASSIMILATION

Initial experiments with leaf discs indicated that the capacity of the disks for photosynthetic CO₂ assimilation was fairly variable. It was then discovered that leaf disks taken from the same plant and opposite leaves exhibited very similar fixation capacities while leaf discs from other plants were quite dissimilar. This held true for plants grown in the same tray right next to each other. To obtain some estimate of the degree of pairing the absolute difference between the dpm for each leaf pair was calculated and the standard deviation found. This was repeated for a random assortment of the same dpm values and the standard deviation for the randomly associated leaf discs was found to be 4.32 times as large as for the paired leaf discs. For this reason substantial replication was required and six replicates consisting of 3 leaf pairs were used for each treatment.

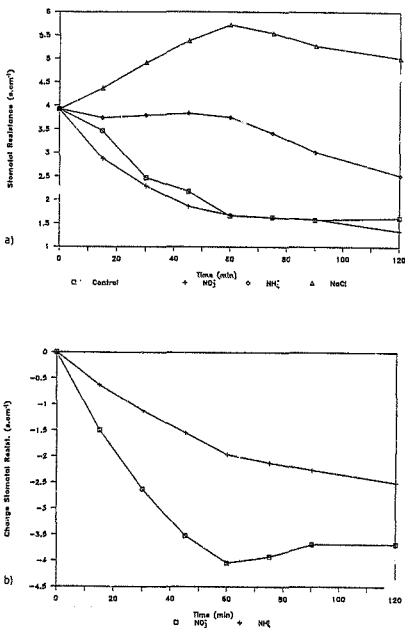


Fig. 17. Stomatal resistance changes in response to the introduction of 10 mM concentrations of various salts into the bathing medium of cut shoots. NO_3^- supplied as NaNO_3 and NH_4^+ as NH_4Cl . Control comprised of plants left in distilled water. Measurements performed 90 minutes after cutting. a) Data normalized around mean starting value. b) Normalized data with NaCl treatment assumed as a reference.

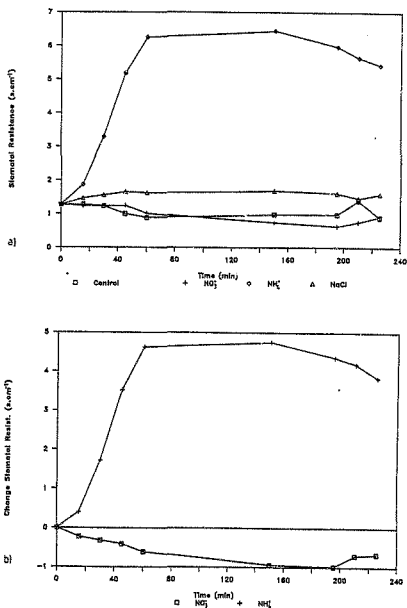


Fig. 18. Stomatal resistance changes in response to the introduction of 20 mM concentrations of various salts into the bathing medium of cut shoots. NO₃⁻ supplied as NaNO₃ and NH₄⁺ as NH₄Cl. Control comprised of plants left in distilled water. Measurements performed 90 minutes after cutting. a) Data normalized around mean starting value. b) Normalized data with NaCl treatment assumed as a reference.

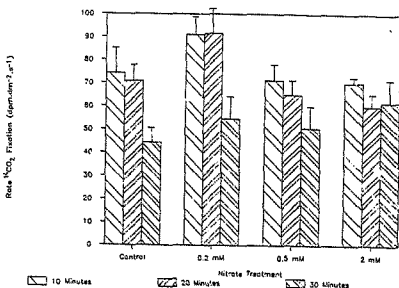


Fig. 19. The influence of NO_3^- on *P. sativum* leaf disc photosynthetic $^{14}\text{CO}_2$ assimilation at various times after infiltration. Bars indicate S.E.

The standard deviation of the results did not exceed 15 % and the average standard deviation was 8.5 %.

The influence of nitrate on CO_2 fixation was investigated and a concentration and time dependent effect established (Fig. 19). With time the rate of $^{14}\text{CO}_2$ assimilation declined, possibly as a result of re-release of $^{14}\text{CO}_2$ from photorespiration. The leaf discs also tended to desiccate partially (10% loss in weight) over the 30 minute period for which the experiment was run and this may have influenced the results. After 10 minutes incubation the leaf discs exposed to 0.2 mM nitrate had accumulated more $^{14}\text{CO}_2$ than the control while higher concentrations exhibited a slightly repressive effect on assimilation. After 20 minutes incubation the pattern was essentially similar to that of 10 minutes while at 30 minutes 2 mM nitrate resulted in the greatest assimilation of $^{14}\text{CO}_2$. Thus it is evident that both the time at which sampling is done and the concentration of nitrate supplied have important ramifications for the effect of this inorganic nitrogen species on photosynthetic CO_2 assimilation.

9.3. PROTOPLAST NITRATE, NITRITE AND AMMONIUM NET UPTAKE

The rates of net uptake of these various nitrogen ionic species were determined in order to gain some quantitative idea of the flux of the various ions into the cells. This was necessary to achieve some perspective on whether the ions were influencing metabolism through their mere presence or whether the metabolism of the ions was more likely to be the cause of the various effects described. The argument may be advanced that the measurements performed determine non-reduced N and not the net uptake of the various nitrogen forms. This possibility does exist, but this would necessitate the leakage of the cellular non-reduced N back into the external medium because, even after rapid centrifugation, a large proportion of the protoplasts, dependent on the initial intactness of the preparation, remained intact as determined by Evans Blue staining.

9.3.1. Nitrate

The assays used for the determination of nitrate net uptake were insensitive to the small changes brought about by protoplast net uptake. The dark anaerobic assay yielded positive results for the utilization of nitrate under these conditions. Nitrite accumulated significantly under these conditions in a concentration dependent fashion. The curve (Fig. 20) followed the general pattern for a concentration dependent enzymatic reaction, but a Lineweaver-Burk plot of this data did not yield the expected straight line and thus no K_m value could be calculated. The NR assay which is dependent on the presence of reduced methyl viologen yielded no nitrite accumulation. No colour developed in any of the samples. This may be the result of the further reduction of nitrite to NH_4^+ through the action of NtR reduced by the methyl viologen.

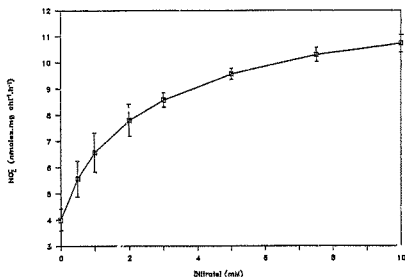


Fig. 20. Nitrate reductase production of NO_2^- in protoplasts under dark anaerobic conditions. Bars indicate S.E.

9.3.2. Nitrite

Net uptake of nitrite was monitored over a 30 minute period in a concentration range 0.01 mM - 0.5 mM (Fig. 21). The rate of net uptake increased markedly over the range 0.01 mM - 0.2 mM but at higher concentrations there was little change. Over a 20 minute period the net uptake of nitrite tended to increase with increasing concentration of nitrite up to 0.2 mM (Fig. 22). At higher concentrations (0.3 mM - 0.5 mM) the rate of net uptake appeared to decline when compared with the rate at 0.2 mM. For the net uptake over 30 minutes no such decline was evident. The rate of net uptake was determined for the various concentrations from the nitrite net uptake which had occurred after 10 minutes (Fig. 23), for the purposes of determining the K_m for net uptake of nitrite. The fact that the rate of

net uptake declined at higher concentrations had some important consequences for the value determined for the K_m . The K_m was determined using a best-fit Lineweaver-Burk plot. Considering all the data points the K_m was found to be 0.2 mM but if the data points of 0.3 mM and 0.5 mM were excluded the K_m was determined to be 0.58 mM.

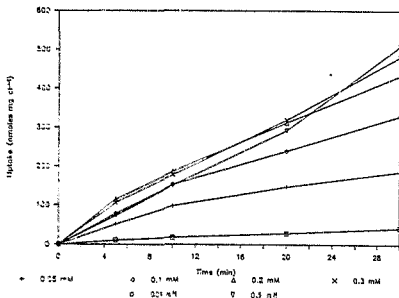


Fig. 21. The uptake of NO_2^- at different exogenous concentrations of this ion over time.

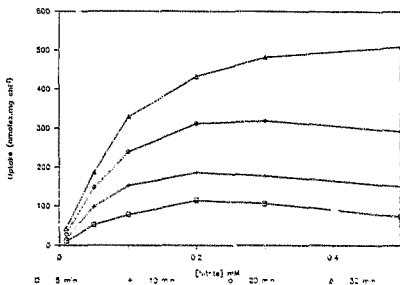


Fig. 22. The concentration dependence of NO_2^- uptake by protoplasts after different times of exposure to NO_2^- .

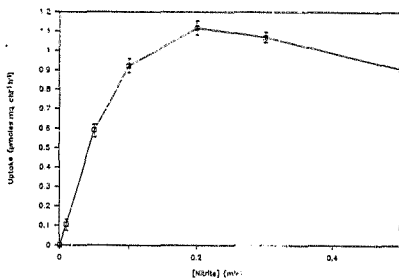


Fig. 23. The kinetics of NO_2^- uptake after 10 minutes of exposure to NO_2^- . The K_m determined from a Lineweaver-Burk plot of this data was 0.62 mM. Bars indicate S.E.

9.3.3. Ammonium

The net uptake of ammonium monitored over a 30 minute time period showed a maximum net uptake when exposed to 0.5 mM ammonium. At concentrations higher than 0.5 mM the rate of net uptake of ammonium was somewhat reduced (Fig. 24). At 2 mM there appeared to be an initial small net uptake of ammonium followed by an efflux from the cells. The net uptake appeared to be continuing after 30 minutes although the rate of net uptake was reduced after 10 minutes for all concentrations as can be seen from the smaller areas between the curves in Fig. 25 after longer time periods.

Initially the rate of net uptake of ammonium increased rapidly with concentration followed by a less rapid decline in net uptake (Fig. 26). The decline was smooth and almost linear with increasing ammonium concentration and finally resulted in an efflux of the ammonium from the protoplasts. The net uptake rates taken from the 10 minute net uptake period were utilized to provide the data for the net uptake K_m calculation. Because of the rapid fall off in net uptake at concentrations higher than 0.5 mM the net uptake K_m was calculated from the data values for concentrations below 0.5 mM. The K_m calculated from a best-fit Lineweaver-Burk plot was 0.039 mM.

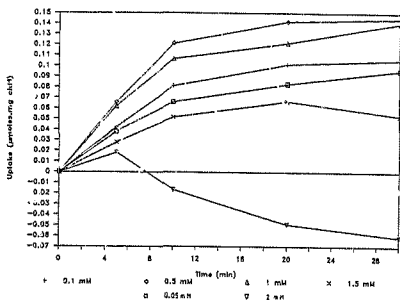


Fig. 24. The uptake of NH_4^+ different exogenous concentration of this ion over time.

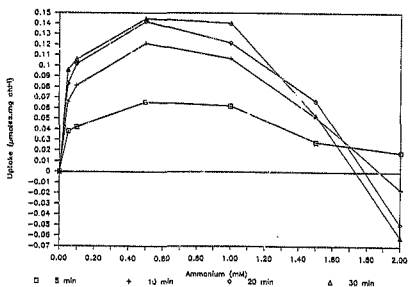


Fig. 25. The concentration dependence of NH_4^+ uptake by protoplasts after different times of exposure to NH_4^+ .

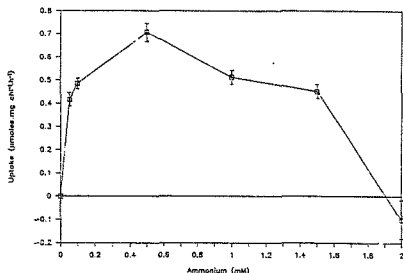


Fig. 26. The kinetics of NH_4^+ uptake after 10 minutes of exposure. The K_m determined from a Lineweaver-Burk plot of this data was 0.039 mM if last three data points were neglected. Bars indicate S.E.

9.4. PROTOPLAST CARBON DIOXIDE ASSIMILATION

$^{14}\text{CO}_2$ assimilation by protoplasts was followed for 45 minutes in the light. The effect of the supply of an inorganic nitrogen was compared against the controls. No effects of NaCl (1 mM) as a control could be established. In these experiments each treatment was replicated three times, including the controls. The results are expressed as the percentage increase from the control of the best-fit linear slope to allow direct comparison of data sets obtained in separate experiments utilizing separate protoplast preparations. The effects of nitrate, nitrite and ammonium were investigated.

9.4.1. Nitrate

The pattern of response of $^{14}\text{CO}_2$ assimilation, measured at a light intensity of $600 \mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, to nitrate showed an initial stimulation of fixation in the concentration range 0 mM - 0.7 mM (Fig. 27a). This was followed at higher concentrations (0.7 mM - 5 mM) by a strong inhibition. The maximum stimulation (19 %) was achieved at 0.16 mM nitrate while the maximal inhibition (23 %) was at the highest concentration examined (5 mM) although the inhibition increased most markedly between 0.7 mM and 1 mM.

At a low light intensity ($200 \mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) the pattern of response was similar to that at $600 \mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ (Fig. 27b and Fig 28). The difference between the two curves was in the range over which the responses occurred and the extent of the responses. The stimulation occurred between 0 mM and 0.1 mM followed by a marked inhibition of $^{14}\text{CO}_2$ assimilation. The stimulatory effect peaked at a lower level of 6.5 %, although the inhibition was similar at the maximum concentration supplied (22 %). From Fig. 28 it appears that the stimulatory and inhibitory effects paralleled for both light intensities.

9.4.2. Nitrite

Nitrite was observed to have a negative effect on $^{14}\text{CO}_2$ assimilation through out the range of concentrations supplied (Fig. 30). The inhibition of $^{14}\text{CO}_2$ assimilation increased most markedly over the range 0 mM - 0.3 mM and thereafter decreased less rapidly with increasing nitrite.

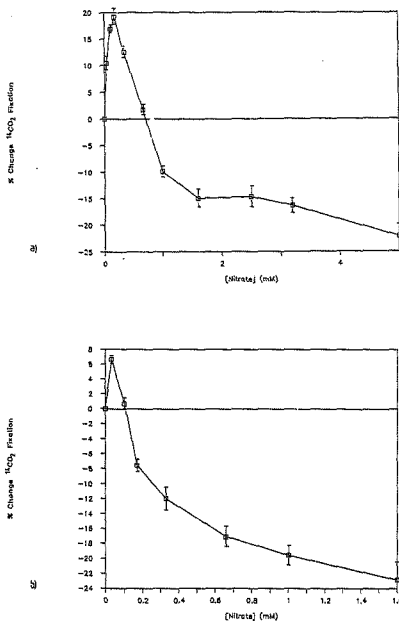


Fig. 27. Response of protoplast CO_2 assimilation to the supply of nitrate at a) high light intensity ($600 \mu\text{E.m}^{-2}\text{s}^{-1}$) b) low light intensity ($200 \mu\text{E.m}^{-2}\text{s}^{-1}$). Bars indicate S.E.

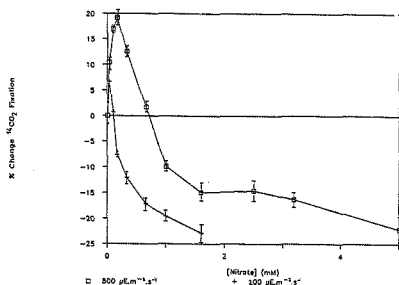


Fig. 28. Comparison of the response of protoplast assimilation to the supply of nitrate at high and low light intensities (and $200 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$). Bars indicate S.E.

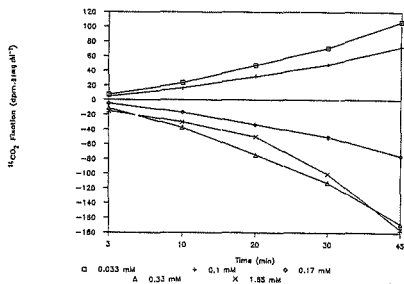


Fig. 29. Raw data (low light intensity) showing the response of protoplast $^{14}\text{CO}_2$ assimilation to the supply of NO_3^- at various concentrations. Initial rates are taken as reference point (0%).

9.4.3. Ammonium

Supply of ammonium had a very short-lived positive effect followed by a very strong inhibition of $^{14}\text{CO}_2$ assimilation (Fig. 31). The stimulatory concentration was 0.033 mM resulting in a 2.4 % increase in assimilation while all other concentrations were inhibitory. The maximum inhibition was 65 % which occurred at the maximum concentration supplied.

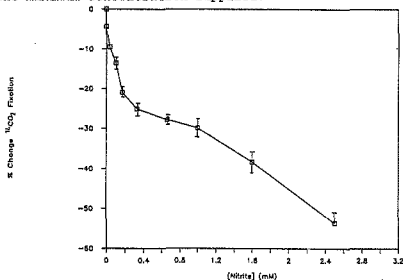


Fig. 30. Response of protoplast CO_2 assimilation to the supply of nitrite. Bars indicate S.E. where larger than symbols.

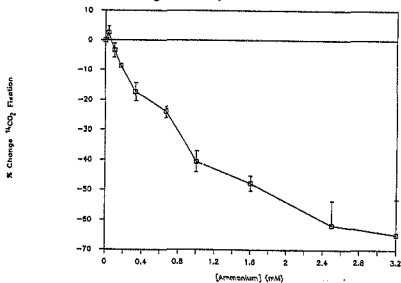


Fig. 31. Response of protoplast CO_2 assimilation to the supply of ammonium. Bars indicate S.E. where larger than symbols.

9.5. PROTOPLAST OXYGEN EVOLUTION

Responses of oxygen evolution by protoplasts incubated with 10 mM NaHCO_3 to various nitrogen salts and sodium phosphate were determined as the deviation from the initial rate of oxygen evolution after the addition of the salts. In order to assess the significance of the changes a statistics test (two phase linear regression analysis using SAS) was run to determine the significance of the changes from the initial rates. All data shown for the influence of the salts on oxygen evolution passed these tests at the $p=0.05$ level at least and in most cases the probability values were $p=0.0001$. As a control to determine whether there were any effects on oxygen evolution due to the addition of a salt to the buffer, the effect of adding NaCl was investigated. This was compared with the effects of nitrate and ammonia at the same concentration (Fig. 32). The slope of the line describing the effect of NaCl on O_2 evolution is seen not to change, and indeed failed the significance test ($p=0.27$) while nitrate and ammonium had significant effects ($p=0.0001$).

Nitrate, nitrite, ammonium and phosphate all had similar concentration dependent effects. The results obtained show a large initial stimulation followed by a decline in the stimulatory effect with increasing concentration (Fig. 33 - Fig. 36).

9.5.1. Nitrate

Nitrate shows an optimal concentration for stimulation of O_2 evolution by 32% at 0.66 mM with a subsequent decrease in stimulation to zero at 3.16 mM at which point further increases in nitrate concentration led to inhibition of O_2 evolution (Fig. 33).

9.5.2. Nitrite

Nitrite had an optimal concentration of 0.66 mM but in contrast to the pattern exhibited by nitrate treated plants the decline in

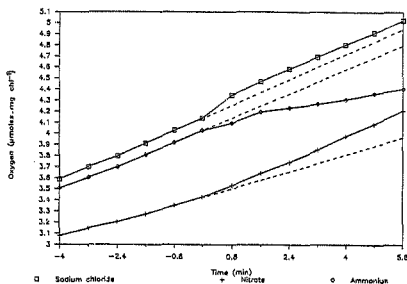


Fig. 32. The responses of pea protoplasts O_2 evolution capacity to the supply of 1.6 mM of three salts ($NaCl$, $NaNO_3$ and NH_4SO_4). The initial slopes are portrayed as starting from -4 minutes to 0 minutes when the various salts were added. The broken lines represent the null hypothesis i.e. no effect of the salts.

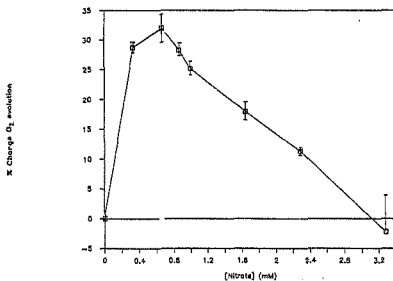


Fig. 33. The influence of NO_3^- concentration on the evolution of O_2 from protoplasts expressed as percent increase above the initial rate prior to the addition of NO_3^- . All values indicated passed a significance test at the $p=0.05$ level. Bars indicate S.E.

stimulation to zero above the optimum concentration only occurred at 6.3 mM (Fig. 34). Thus the range of concentrations over which nitrite was stimulatory was much broader than that for nitrate.

9.5.3. Ammonium

The effect of ammonium was somewhat different to the previous two nitrogen salts in that the stimulatory effect was very much more transient (Fig. 35). The greatest stimulation (10 %) occurred at 0.33 mM with a subsequent inhibition of up to 82 % at the highest concentration supplied (1.66 mM).

9.5.4. Phosphate

The pattern of response to phosphate (supplied as Na_3PO_4) showed an optimum response at 0.86 mM with a subsequent decline in stimulation to zero at 2.4 mM becoming inhibitory at higher concentrations (Fig. 36).

9.5.5. Sodium bicarbonate

The effect of various concentrations of NaH_2CO_3 on O_2 protoplast evolution were also determined. In the experiments in which the effects of nitrate, nitrite, ammonium and phosphate were investigated the various concentrations of the salts were added to a fresh preparation of protoplasts in each case. In the experiments performed to investigate the effects of bicarbonate, various concentrations of the various substances were added sequentially to the same preparation. This was done to reduce the variability between data and to allow the testing of combinations of various treatments. For this reason 'best-fit' linear regression was performed on each portion of a curve corresponding to a treatment. The 'best-fit' lines were then plotted and the correlation coefficients quoted with each line. The slopes of these lines were also plotted to allow ready assessment of the effects of the

various treatments.

Without the addition of NaH_2CO_3 net oxygen evolution did not occur, or occurred at very low rates ($0 - 3.9 \mu\text{moles.mg chl}^{-1}.\text{h}^{-1}$) (Fig. 37a). The pre-treatment of the protoplasts was found to be significant in that protoplasts pre-incubated in the light did not evolve oxygen while those pre-incubated in the dark did. In the experiments investigating the effect of bicarbonate on O_2 evolution, the protoplasts were pre-incubated in the light to prevent the possibility of CO_2 accumulation within the protoplast suspension.

On addition of low concentrations of bicarbonate the rate of oxygen evolution was dramatically increased (Fig. 37a). Bicarbonate continued to enhance O_2 evolution up to a concentration of 15 mM after which there was a decline in the enhancement with increasing concentration although O_2 evolution still occurred at greater rates than those exhibited by CO_2 depleted systems (Fig. 37b and Fig. 37c).

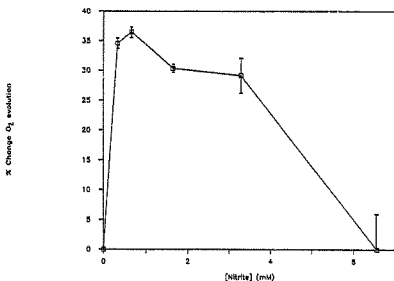


Fig. 34. The influence of NO_2^- concentration on the evolution of O_2 from protoplasts expressed as percent increase above the initial rate prior to the addition of NO_2^- . All values indicated passed a significance test at the $p=0.05$ level. Bars indicate S.E.

various treatments.

Without the addition of NaH_2CO_3 net oxygen evolution did not occur, or occurred at very low rates ($0 - 3.9 \mu\text{moles.mg chl}^{-1}.\text{h}^{-1}$) (Fig. 37a). The pre-treatment of the protoplasts was found to be significant in that protoplasts pre-incubated in the light did not evolve oxygen while those pre-incubated in the dark did. In the experiments investigating the effect of bicarbonate on O_2 evolution, the protoplasts were pre-incubated in the light to prevent the possibility of CO_2 accumulation within the protoplast suspension.

On addition of low concentrations of bicarbonate the rate of oxygen evolution was dramatically increased (Fig. 37a). Bicarbonate continued to enhance O_2 evolution up to a concentration of 15 mM after which there was a decline in the enhancement with increasing concentration although O_2 evolution still occurred at greater rates than those exhibited by CO_2 depleted systems (Fig. 37b and Fig. 37c).

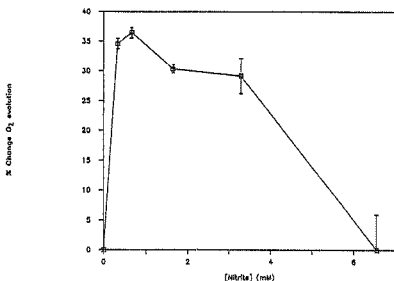


Fig. 34. The influence of NO_2^- concentration on the evolution of O_2 from protoplasts expressed as percent increase above the initial rate prior to the addition of NO_2^- . All values indicated passed a significance test at the $p=0.05$ level. Bars indicate S.E.

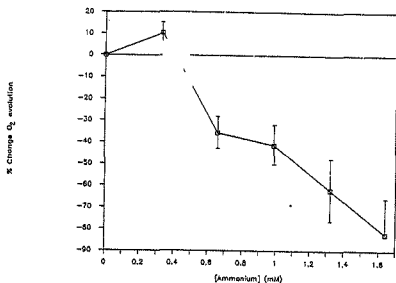


Fig. 35. The influence of NH_4^+ concentration on the evolution of O_2 from protoplasts expressed as percent increase above the initial rate prior to the addition of NH_4^+ . All values indicated passed a significance test at the $p=0.05$ level. Bars indicate S.E.

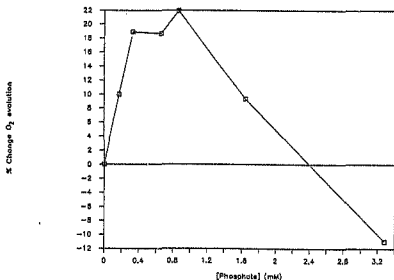


Fig. 36. The influence of PO_4^{2-} concentration on the evolution of O_2 from protoplasts expressed as percent increase above the initial rate prior to the addition of PO_4^{2-} . All values indicated passed a significance test at the $p=0.05$ level. Insufficient replication was performed to allow calculation of S.E.

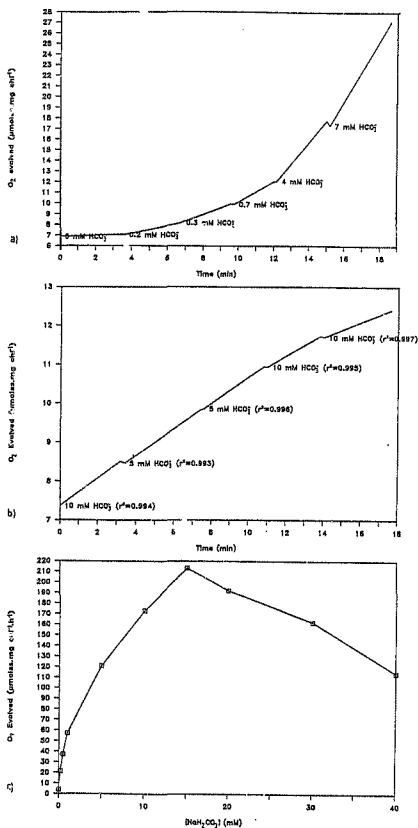


Fig. 37. Changes in oxygen evolution in response to sequential additions of $NaHCO_3$. a) and b) Concentration O_2 in protoplast suspension. c) Rate O_2 evolution as influenced by additions.

9.5.5.1. Interaction between Nitrate and Sodium Bicarbonate

A combination of four different NaHCO_3 concentrations and various concentrations of NaNO_3 were investigated. Pre-incubation of the protoplasts was performed in the dark to retain constancy with the experimental procedure used during the investigation of the effects of nitrate, nitrite, ammonium and phosphate on O_2 evolution by protoplasts incubated in 10 mM NaHCO_3 . Concentrations of NO_3^- were selected to fall in ranges expected to stimulate and inhibit oxygen evolution.

Protoplasts not supplied with added NaHCO_3 evolved oxygen at significant rates ($14.8 \mu\text{moles O}_2 \cdot \text{mg chl}^{-1} \cdot \text{h}^{-1}$). The addition of 1 mM NO_3^- resulted in no change or a very slight stimulation of O_2 evolution (Fig. 38) while addition of a further 4 mM NO_3^- resulted in a strong inhibition of O_2 evolution. This effect was found to be reversible with the addition of 1 mM NaHCO_3 which enhanced O_2 evolution above the initial rate.

Protoplasts incubated in 1 mM NaHCO_3 showed a small stimulation of oxygen evolution in the presence of 1 mM NO_3^- (12%) while addition of a further 3 mM NO_3^- decreased O_2 evolution (Fig. 39) but not by as much as in the case of protoplasts not supplied with NaHCO_3 (Fig. 38). Again the addition of further bicarbonate was able to reverse the effects of high NO_3^- concentrations, but the level of O_2 evolution was not enhanced above the initial rate in this case.

Protoplasts incubated with 5 mM NaHCO_3 showed a large stimulation (37%) in response to 1 mM NO_3^- (Fig. 40). Further addition of 3 mM NO_3^- resulted in a maintenance of this enhancement effect (26%) as did a further 3 mM NO_3^- (10%) but at concentrations exceeding this (addition of a further 6 mM NO_3^-) resulted in a suppression of O_2 evolution.

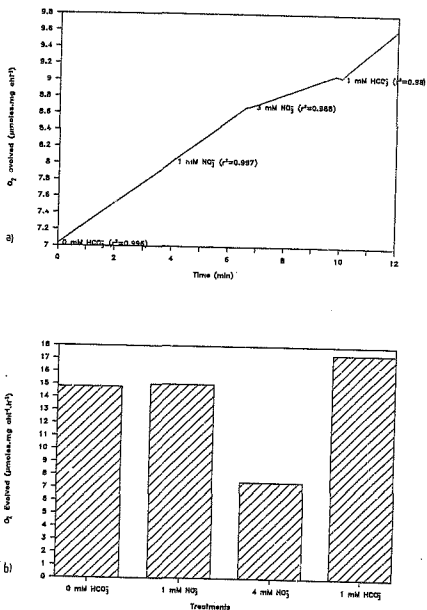


Fig. 38. Changes in oxygen evolution by protooplasts incubated without NaHCO₃ in response to different concentrations of NO₃⁻. a) O₂ concentration in protooplasts suspension. b) Rate of O₂ evolution as influenced by additions.

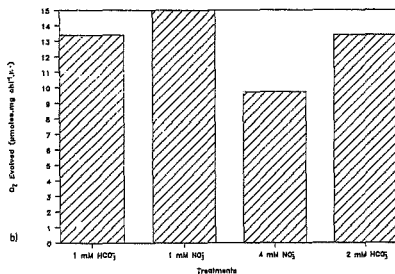
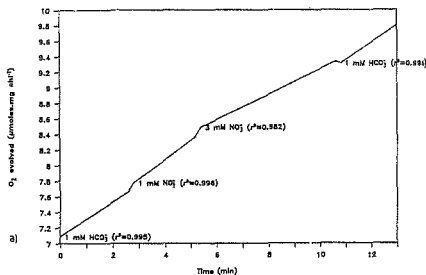


Fig. 39. Changes in oxygen evolution by protooplasts incubated in 1 mM NaHCO₃ in response to different concentrations of NO₃⁻. a) O₂ concentration in protooplasts suspension. b) Rate of O₂ evolution as influenced by additions.

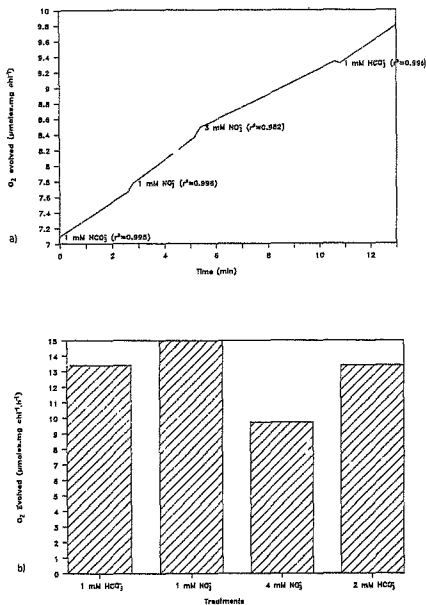


Fig. 39. Changes in oxygen evolution by proto-plasts incubated in 1 mM NaHCO₃ in response to different concentrations of NO₃⁻. a) O₂ concentration in proto-plasts suspension. b) Rate of O₂ evolution as influenced by additions.

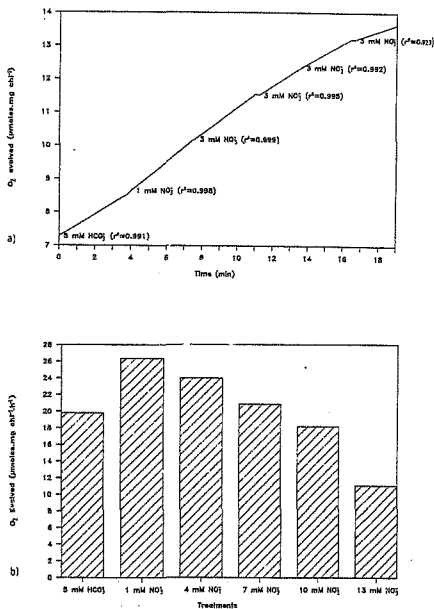


Fig. 40. Changes in oxygen evolution by proto-plasts incubated in 5 mM NaHCO_3 in response to different concentrations of NO_3^- . a) O_2 concentration in proto-plasts suspension. b) Rate of O_2 evolution as influenced by additions.

9.5.5.2. Interaction between Ammonium and Sodium Bicarbonate

A combination of three different NaHCO_3 concentrations and various concentrations of NH_4Cl were investigated. Pre-incubation of the protoplasts was performed in the dark. Concentrations of NH_4^+ were selected to fall in ranges expected to stimulate and inhibit oxygen evolution.

In the absence of NaHCO_3 the effects of ammonium could not be distinguished from the variability of the data and attempts to perform linear regression on this data resulted in small correlation coefficients. In the presence of 1 mM NaHCO_3 , 1 mM NH_4^+ increased O_2 evolution significantly (42%) (Fig. 41). Further addition of NH_4^+ (3 mM) inhibited O_2 evolution and this effect was not reversible with even very high concentrations of bicarbonate (10 mM).

Oxygen evolution by protoplasts incubated with 5 mM NaHCO_3 was stimulated (27%) by addition of 1 mM NH_4^+ while higher concentrations resulted in an inhibition of O_2 evolution (Fig. 42). The stimulatory effect of 1 mM NH_4^+ was reduced in comparison to that observed for protoplasts incubated in 1 mM NaHCO_3 (Fig. 41).

Oxygen evolution by protoplasts incubated with 10 mM NaHCO_3 was stimulated (6%) by addition of 1 mM NH_4^+ while higher concentrations resulted in an inhibition of O_2 evolution (Fig. 43). The stimulatory effect of 1 mM NH_4^+ was reduced in comparison to that observed for protoplasts incubated in 5 mM NaHCO_3 (Fig. 42).

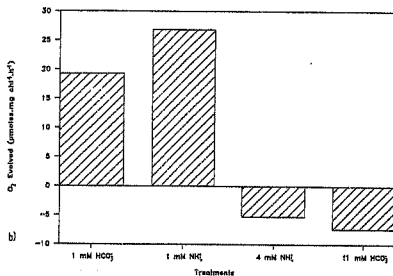
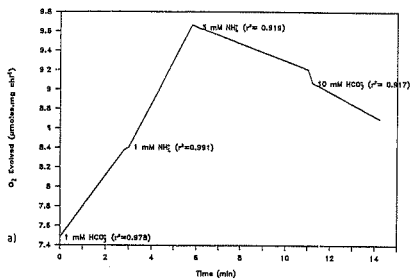


Fig. 41. Changes in oxygen evolution by protooplasts incubated in 1 mM NaHCO₃ in response to different concentrations of NH₄⁺. a) O₂ concentration in protooplasts suspension. b) Rate of O₂ evolution as influenced by additions.

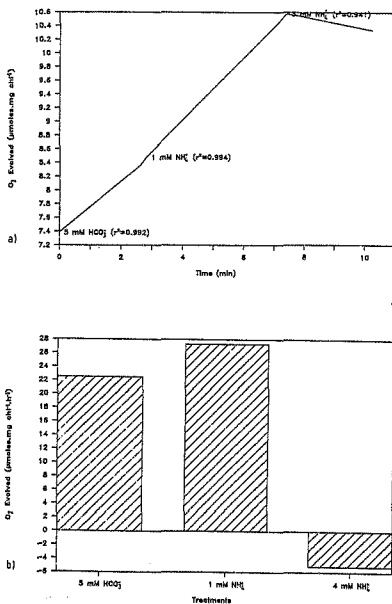


Fig. 42. Changes in oxygen evolution by protozoa incubated in 5 mM $NaHCO_3$ in response to different concentrations of NH_4^+ . a) O_2 concentration in protozoa suspension. b) Rate of O_2 evolution as influenced by additions.

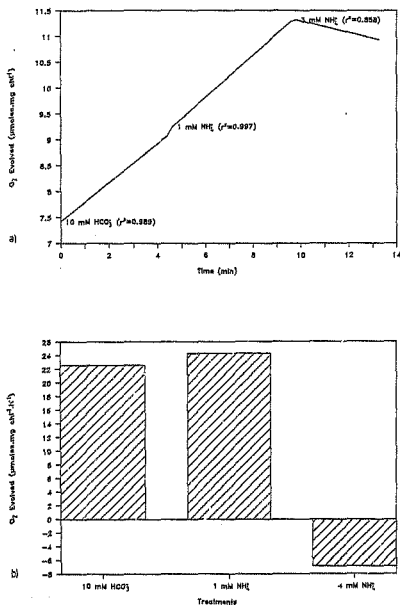


Fig. 43. Changes in oxygen evolution by protoplasts incubated in 10 mM $NaHCO_3$ in response to different concentrations of NH_4^+ . a) O_2 concentration in protoplasts suspension. b) Rate of O_2 evolution as influenced by additions.

9.5.5.3. The effects of respiratory inhibitors

The effects of monofluoroacetic acid (MFA) in combination with KCN and various concentrations of NO_3^- were investigated. The protoplasts were pre-incubated in the dark. The MFA was either injected into the incubation medium during the assay or the protoplasts were pre-incubated in 1 mM MFA for 1 hour prior to the assay. MFA (Sigma) was used as an inhibitor of respiration. This substance substitutes for acetate in the TCA cycle and may thus inhibit respiration at least partially (Elrifí and Turpin, 1986).

In the presence of 10 mM NaHCO_3 , 1 mM MFA enhanced O_2 evolution (49%) and further additions of MFA up to a final concentration of 10 mM did little to alter this stimulation (Fig. 44). KCN (about $0.003\text{g}\cdot\text{ml}^{-1}$) caused an immediate cessation of O_2 evolution and rapid O_2 consumption. In the dark this rate of O_2 consumption was considerably reduced (68%) while return to light conditions resulted in a slight increase in the rate of O_2 consumption.

Cells pre-incubated in 1 mM MFA without added NaHCO_3 appeared to evolve O_2 at a low rate ($1\text{ }\mu\text{mole O}_2\cdot\text{mg chl}^{-1}\cdot\text{h}^{-1}$) (Fig. 45a) while protoplasts not pre-incubated in MFA exhibited no net O_2 evolution (Fig. 45b). In both cases 1 mM NaHCO_3 resulted in a stimulation of O_2 evolution. Nitrate (1 mM) also stimulated O_2 evolution in both cases. Additional 3 mM NO_3^- decreased O_2 evolution (10%) by the MFA treated cells but less than shown previously for non-MFA treated cells (Fig. 39). A comparison of the MFA pre-incubated cells and those not pre-incubated in MFA (Fig. 45c) indicates that MFA pre-incubation dramatically increased O_2 evolution and that the responses of cells incubated in MFA were greater (19%) than those of normal cells (10% - 11%).

Cells pre-incubated in MFA and incubated with 10 mM NaHCO_3 showed enhanced O_2 evolution with 1 mM NO_3^- but a further 3 mM NO_3^- decreased O_2 evolution (Fig. 46).

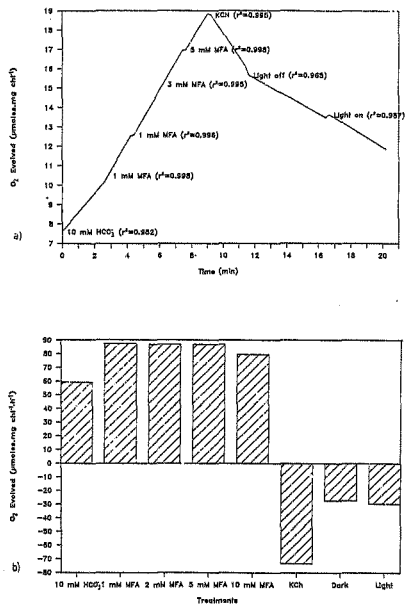


Fig. 44. Changes in oxygen evolution by protooplasts incubated in 10 mM NaHCO₃ in response to different concentrations of MFA and KCN. a) O₂ concentration in protooplasts suspension. b) Rate of O₂ evolution as influenced by additions.

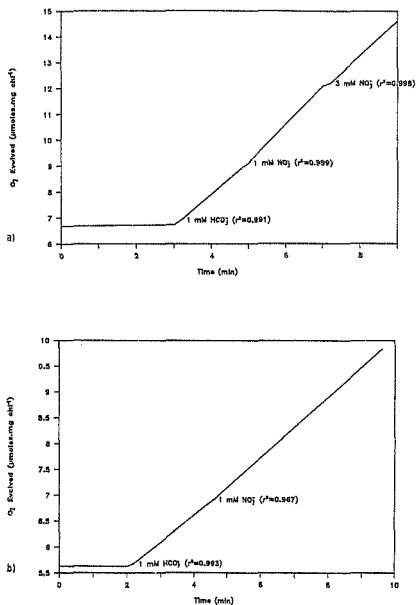


Fig. 45. Changes in oxygen evolution by protoplasts in response to different concentrations of NaHCO_3 and NO_3^- . a) O_2 concentration in protoplasts suspension incubated in 1 mM MFA. b) O_2 concentration in protoplasts suspension not incubated in MFA.

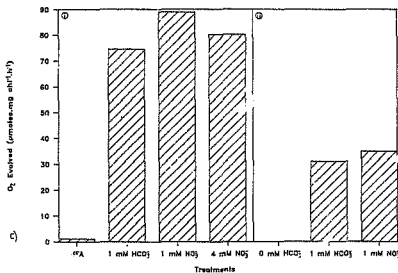


Fig. 45 (cont.) c) i) Rate of O₂ evolution by proto-plasts incubated in 1 mM MFA as influenced by additions ii) Rate of O₂ evolution by proto-plasts not incubated in MFA as influenced by additions.

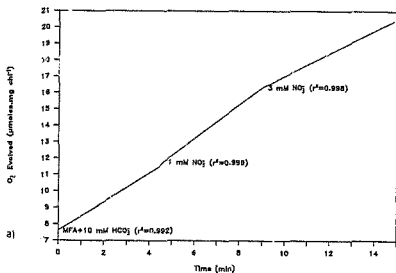


Fig. 46. Changes in oxygen evolution by proto-plasts incubated in 1 mM MFA and 10 mM NaHCO₃ in response to different concentrations of NO₃⁻. a) O₂ concentration in proto-plasts suspension.

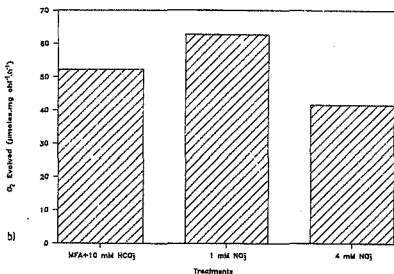


Fig. 46 . (cont.) b) Rate of O₂ evolution as influenced by additions.

9.6. THE EFFECT OF NITRATE ON PROTOPLAST ATP LEVELS

The response of ATP levels in the protoplasts was examined at levels of NO₃⁻ chosen to represent the concentrations found to be inhibitory and stimulatory on CO₂ fixation. The changes in the levels of ATP are expressed as a percentage change from the control levels and the results graphed in Fig. 47 are the means of 3 determinations. Initially the levels of ATP were depressed at both concentrations, but the low NO₃⁻ treatment (0.17 mM) recovered rapidly and the ATP levels rose above the initial level. At the higher concentration (1 mM) the levels did recover to some extent, but did not exceed the starting levels.

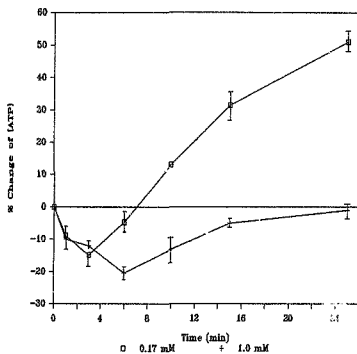


Fig. 47. Response of ATP levels in protoplasts to the supply of NO_3^- at two concentrations. Low concentration (0.17 mM) selected for its stimulatory effect while 1 mM exhibited an inhibitory effect on CO_2 evolution. Percentage change calculated as deviation from the control.

10. DISCUSSION

10.1. SHOOT PHOTOSYNTHETIC CARBON ASSIMILATION

The long term effects of NO_3^- on photosynthesis may be attributable to a large number of phenomena (Hunt et al., 1985a;b;c). The main possibilities are listed below.

- a) N nutrition may cause an increase in general protein synthesis and thus result in generally increased metabolic activity.
- b) The activity of the photosynthetically linked enzymes may be altered.
- c) The activity of the photorespiratory enzymes and of the photorespiratory pathway may be affected.
- d) Respiratory metabolism may be moderated.
- e) Uptake, translocation and transport of inorganic N may place additional demands on carbon metabolism resulting in a greater demand for photosynthate.
- f) The reduction and carbon requirements for NO_3^- and NO_2^- reduction and NH_4^+ assimilation may also form a sink for photosynthate or compete with this process.
- g) Inorganic nitrogen may have effects on the transport and metabolism of other ions and compounds not related to the reduction requirements of the inorganic nitrogen ions per sé.
- f) Stomatal conductance may be influenced.

An increase in the the levels RuBPC and glyceraldehyde phosphate dehydrogenase in response to nitrogen has been reported (Avdreeva and Andreeva, 1973) but whether the reported increases in the ac-

tivity of these enzymes is specific or related to a general elevation of protein synthesis is questionable. The activities of RuBPO (Tew et al., 1974; Cresswell et al., 1977), glycolate oxidase, catalase (Fair et al., 1974), RuBPC and PEPc (Tew, 1976) have however been reported to increase with nitrate and ammonium nutrition in several species of grass. Thus one would expect photorespiratory activity to increase (Cresswell et al., 1977) which would result in a decrease in net photosynthetic activity (Tew, 1976). Two possibilities for the effect of inorganic nitrogen on enzyme activities exist. Either the nitrogen may regulate the level of the enzymes or the nitrogen may affect the activity of the enzyme directly. The latter alternative has been favoured for the changes in ratio between RuBPC/RuBPO but no kinetic evidence exists for such mechanisms. Low compensation points in N fed barley have recently been attributed to alteration in mitochondrial activity (Marek and Frank, 1984) and thus respiratory decreases may mediate increased net photosynthetic rates. Uptake of inorganic nitrogen has been reported to be active for some of the ionic forms (See Section 7.1.2.). Translocation is likely to involve xylem conduction from the root to the shoot. Transport across cellular membranes may be passive (NO_2^-) or involve some form of transport system. The importance of reductant and carbon provision will be discussed elsewhere.

10.1.1. *Lycopersicon esculentum*

10.1.1.1. Pre-treatment effects

Associated with chlorosis and stunted shoots of plants starved of nitrogen was a low net photosynthetic rate in comparison to plants grown with the normal quantity of nitrogen in the form of NO_3^- (14.8 mM). Supply of N nutrition (as NO_3^- in Long Ashton medium) elevated the photosynthetic rate regardless of how long the supply occurred before the photosynthetic rate was determined (Fig. 6). Considering that the growth medium was flushed daily with distilled water, this may imply that after initial exposure to N nutrition, the effect of that nutrition was retained for

some time.

The effects of inorganic nitrogen on net photosynthetic carbon assimilation through uptake, translocation, transport, reduction and effects on other ions are undoubtedly a possibility and there is evidence for all these effects in the literature. Over the time periods for which the results under discussion were obtained, there is no way of eliminating any of these effects. It is likely that the response of the net photosynthetic CO_2 assimilation is a result of a combination of many factors.

It is however noteworthy that the response to N nutrition was relatively small within a few hours of application with greater responses a number of days after application to nitrogen starved plants. Thus it appears that the effects were slow in becoming manifest and some synthetic mechanism is likely to be important. The transition from N free medium to N supply may initiate general protein synthesis and facilitate the use of stored carbohydrates for provision of reductant and carbon skeletons for nitrate reduction to amino acids. The time between supply and maximal photosynthesis may thus represent the time taken for the plant to recover from the N limited condition. That the plants released from N limited conditions exhibited greater photosynthetic rates than those plants regularly supplied with N may indicate the greater demands for photosynthate resulting from such a recovery. The slightly lower photosynthetic rate 4 days after supply in comparison to the rate 3 days after supply may result from a re-initiation of the N limited condition, or the depletion of substrate and plant reserves of inorganic N which may be important for the maintenance of maximal photosynthesis. An alternative possibility is that either respiration or photorespiration was stimulated at this point but there is no likely explanation as to why this should have occurred after such a long delay.

The short term effects of NO_3^- on net photosynthetic CO_2 assimilation (Fig. 7) of tomatoes was characterized by the largest response occurring in plants fed NO_3^- 1.6 days prior to measurement. This may result from the system being most fully induced for metabolism of NO_3^- at this stage. Although induction of

NR is known to occur in maize within a few hours of supply, full induction may only be achieved after 8 hours or more (Gray, 1984). The transition from a N starved situation to one of sufficient N may be a longer term response involving induction of the photosynthetic systems. Normal N fed plants exhibited an intermediate response. Plants most recently supplied with NO_3^- (0.4 days) exhibited only a transient response to the ions. This response occurred over a short time interval and may be related to the uptake, transport, translocation or reduction requirements of the ions. These plants were probably saturated with NO_3^- at the time of measurement and thus were relatively insensitive to further additions. The lack of a significant lag phase in this and other experiments suggests that translocation of the ions to the leaf tissue was very rapid and that the effects of the NO_3^- were not related to a general elevation of protein synthesis.

10.1.1.2. The influence of nitrate and ammonium

In plants adequately supplied with N nutrition the short term response of net photosynthetic CO_2 assimilation to NO_3^- was concentration dependent (Fig. 8). The low concentration stimulation of net photosynthetic CO_2 assimilation and a high concentration dependent inhibition was a characteristic pattern found throughout this investigation. The response of net CO_2 fixation showed a maximum stimulation when shoots were introduced into a 0.2 mM NO_3^- bathing medium (Fig. 9). The mechanism of these effects may be related to any one of, or subset of, the possibilities listed above. Considering the extremely short lag between supply and response, it is unlikely that synthesis was required for the response. Because uptake was through a cut surface the possibility of uptake effects is eliminated. The remaining alternatives are that NO_3^- transport into the cell or vacuole places indirect demands on the photosynthetic system or that the cytoplasmic reduction requirements of this ion, or its products, in some way enhances CO_2 assimilation.

Ammonium stimulation of net photosynthetic CO_2 assimilation (Fig. 10) may be attributable to possible direct effects of the ion on

enzyme activity. Ammonium stimulation of photorespiration in the long term has been reported by other workers (Tew, 1976). Exogenous NH_4^+ is normally assimilated in the root and translocated to the shoot as amino acids (Lewis and Chadwick, 1983), although there is extensive release and reassimilation of NH_3 through the photorespiratory nitrogen cycle. Blockage of photorespiratory nitrogen reassimilation (with MSO) in Themeda triandra resulted in an inhibition of net photosynthetic CO_2 assimilation (Amory and Cresswell, 1986). This decrease was correlated to an increased CO_2 compensation point which was ascribed to the operation of an alternative photorespiratory pathway involving formate. In this study the effects of ammonium on CO_2 compensation points were not examined, but the results of Amory and Cresswell (1986) indicate that photosynthetic activity would have to increase against increased photorespiratory CO_2 release. In the experimental system employed here the root was absent and thus translocation and transport of NH_4^+ could place demands on the Calvin cycle for provision of carbon. Metabolism of ammonium requires carbon skeletons for the formation of amino acids. The amino acid products of ammonium assimilation may have requirements for transport, translocation and further metabolism which may depend on carbon metabolism for energy provision.

10.1.2. *Pisum sativum*

10.1.2.1. Pre-treatment effects

Starvation of *Pisum sativum* had totally different consequences to similar treatment of *Lycopersicon esculentum*. The lack of visible signs of N starvation, apart from very slight chlorosis, in young plants was correlated with a lack of a significant difference in net photosynthetic assimilation rates between N fed and starved plants (Fig. 11). This difference between the responses of the two species is probably due to the difference in the size of the seeds. The large *P. sativum* seeds were observed to be sufficient to maintain vigorous growth in albino mutants for up to 14 days. No nodulation was evident on the roots of *P. sativum* and thus N_2 -

fixation is unlikely to be a factor. In addition the growth rates of the peas were more rapid and these plants were used at a younger age than the tomatoes. Thus the effects of N starvation were not as severe in the case of the former. Another factor related to species was the magnitude of the photosynthetic rates. From comparison of the data for tomato and pea net photosynthetic rates it appears that the former functions more efficiently in net photosynthetic CO_2 assimilation ($13.2 \text{ mg} \cdot \text{dm}^{-2} \cdot \text{h}^{-1}$ versus $5.2 \text{ mg} \cdot \text{dm}^{-2} \cdot \text{h}^{-1}$).

The stimulatory effect of N nutrition 3 hours prior to determination of the photosynthetic rates may once again be attributed to a number of factors. The lack of a significant difference between N starved and N fed plants implies that the photosynthetic system, and probably other metabolic systems, in the starved plants were as active as those of the N starved plants and thus induction of a synthetic phase for recovery from starvation was not required. In addition the lack of significant change in plants supplied 17 hours prior to photosynthetic rate determination indicates that any change which N nutrition may have brought about in general anabolism was minimal. Thus the remaining possibilities include the effects of inorganic N (direct and indirect) on enzyme levels/activities, uptake, translocation, transport and reduction.

The reference against which photosynthetic rates were determined was found to be of some significance. The above results are expressed on the basis of leaf area. If the results are expressed on the basis of chlorophyll concentration a somewhat different picture emerges. The distinctions between these two expressions results from the differential effects of N nutrition on leaf area and chlorophyll content. In peas N starvation did not appear to alter the photosynthetic rates per leaf area which means that plant photosynthetic capacity was linear with leaf area. However chlorophyll content was altered through N starvation and the plants appeared slightly chlorotic. Photosynthetic capacity is however not linearly correlated with chlorophyll content and thus an increase in chlorophyll content (N fed plants) results in a decrease in photosynthetic rate when expressed on the basis of

fixation is unlikely to be a factor. In addition the growth rates of the peas were more rapid and these plants were used at a younger age than the tomatoes. Thus the effects of N starvation were not as severe in the case of the former. Another factor related to species was the magnitude of the photosynthetic rates. From comparison of the data for tomato and pea net photosynthetic rates it appears that the former functions more efficiently in net photosynthetic CO_2 assimilation ($13.2 \text{ mg.dm}^{-2}.\text{h}^{-1}$ versus $5.2 \text{ mg.dm}^{-2}.\text{h}^{-1}$).

The stimulatory effect of N nutrition 3 hours prior to determination of the photosynthetic rates may once again be attributed to a number of factors. The lack of a significant difference between N starved and N fed plants implies that the photosynthetic system, and probably other metabolic systems, in the starved plants were as active as those of the N starved plants and thus induction of a synthetic phase for recovery from starvation was not required. In addition the lack of significant change in plants supplied 17 hours prior to photosynthetic rate determination indicates that any change which N nutrition may have brought about in general anabolism was minimal. Thus the remaining possibilities include the effects of inorganic N (direct and indirect) on enzyme levels/activities, uptake, translocation, transport and reduction.

The reference against which photosynthetic rates were determined was found to be of some significance. The above results are expressed on the basis of leaf area. If the results are expressed on the basis of chlorophyll concentration a somewhat different picture emerges. The distinctions between these two expressions results from the differential effects of N nutrition on leaf area and chlorophyll content. In peas N starvation did not appear to alter the photosynthetic rates per leaf area which means that plant photosynthetic capacity was linear with leaf area. However chlorophyll content was altered through N starvation and the plants appeared slightly chlorotic. Photosynthetic capacity is however not linearly correlated with chlorophyll content and thus an increase in chlorophyll content (N fed plants) results in a decrease in photosynthetic rate when expressed on the basis of

chlorophyll. Changes in chlorophyll content are also more likely to occur more rapidly than leaf expansion and thus the stimulation of photosynthesis within 3 hours of N nutrition was more significant when expressed on the basis of leaf area than chlorophyll content. This would suggest that the chlorophyll was sufficient even in chlorotic plants to maintain CO_2 fixation unless any increase in photosynthetic activity, with increased chlorophyll content, was correlated with increased photorespiratory activity.

10.1.2.2. The influence of nitrate and ammonium

A similar effect to that in tomato was observed in response to the short term provision of NO_3^- to N fed plants (Fig. 12). Low concentrations of NO_3^- stimulated net photosynthetic assimilation (Fig. 14). The stimulation observed in pea was considerably smaller than that of tomato and the concentration range over which a positive effect was observed was much wider. This, coupled with lower photosynthetic rates, may imply that the pea photosynthetic system was functioning closer to its maximum limit. Nitrate can only increase the photosynthetic rate up to some maximum limit; a limit which may be determined by factors unrelated to the effects of NO_3^- supply. The short lag phase prior to stimulation of net CO_2 fixation may be attributable to the time required for translocation to the leaf tissue. This short lag suggests that synthesis of proteins was not important for this response. That the effect of NO_3^- became inhibitory at higher concentrations makes it unlikely that this ion would play a direct rôle in the modulation of photosynthetic enzyme activity. This leaves the possibility that the ionic influence was exerted through transport, photorespiratory, respiratory or reduction mechanisms described previously.

Supply of NH_4^+ also resulted in a concentration dependent effect in pea (Fig. 13). The effects of NH_4^+ may be related to direct effects on enzyme levels/activities although the considerations described above for NO_3^- apply equally here. The absence of requirements for uptake and translocation of NH_4^+ was discussed

previously. The reduction of NH_4^+ in the leaf is known to be chloroplastic light reaction dependent (Atkins and Calvin, 1975; Anderson and Walker, 1983) and thus is unlikely to affect photosynthetic CO_2 fixation directly. The assimilation of NH_4^+ into amino acids does however require carbon skeletons which may be provided directly or indirectly from the Calvin cycle and may thus moderate CO_2 assimilation. Thus, if the light reaction has the capacity to supply sufficient reducing equivalents for the assimilation of both CO_2 and NH_4^+ , then the NH_4^+ may enhance CO_2 assimilation. If the concentration of ammonium is increased the withdrawal of reducing potential and ATP from the light reaction may become competitive with CO_2 assimilation. Another possibility is that at higher concentrations, NH_4^+ uncouples photophosphorylation and/or inhibits the functioning of the light reaction (Izawa, 1977) which would result in diminished CO_2 assimilation. This may explain the concentration dependent effects of NH_4^+ on CO_2 assimilation (Fig. 14) although the rôle of photorespiration in NH_4^+ assimilation and CO_2 metabolism cannot be ignored.

10.1.2.3. Carbon dioxide compensation points

The interpretation of compensation point data is complex as a result of the interaction of photosynthesis, photorespiration and respiration influencing the magnitude of this parameter. Compensation points represent the CO_2 concentration at which photosynthetic assimilation balances photorespiratory and respiratory disassimilation at a given light intensity. The compensation point is in any case an underestimation of CO_2 disassimilation as a consequence of the possibility of photosynthetic reassimilation of CO_2 . Because the balance point occurs at a relatively low CO_2 concentration (0-100 ppm), the rates at which these processes occur is unlikely to reflect their true rates under normal conditions because respiration is likely to be favoured by a steeper gradient of CO_2 while photosynthesis will be reduced for the same reason. The ratio of CO_2/O_2 would also be increased and thus photorespiration would be stimulated. Any modification of the

compensation point may be attributed to a change in any one of the participating factors. Considering that we have information about the response of net photosynthesis to various treatments it is possible to obtain at least an estimate of the activity of photorespiration, although net photosynthesis is itself modified by disassimilatory activity. It was previously believed that respiratory CO_2 evolution was only a small component of the CO_2 evolved by the combination of photorespiration and respiration. This may be true to some extent although work by Marek and Frank (1983) indicates that respiration may be an important component of the compensation point.

Results obtained in this investigation indicate that N deficiency resulted in an increase of compensation point (Fig. 15). This is in agreement with the results of Frank and Marek (1983) who found a similar trend in barley. In contrast the compensation points were elevated 17.3 hours after N nutrition. This elevation in compensation point was associated with a small increase in photosynthetic capacity. Thus the gross photosynthetic capacity must have changed more than suggested by net photosynthesis.

Due to the complexities of this system it is difficult to speculate on the possible mechanism of these effects. It is possible that the elevation of compensation point in plants transferred to N nutrition from N limited conditions was the result of NO_3^- reduction utilizing reducing potential derived from the metabolism of glycolate to glyoxylate as suggested by Lips (1971). Although an elevation of the compensation points occurred with N nutrition of the intact plants, no such elevation was evident in cut shoots supplied with NO_3^- over the period of an hour. This would seem to negate the proposal that the effect was as a result of the requirements resulting from nitrate reduction. In changing from a N limited condition amino acids may become available facilitating photorespiratory activity, or transition to N sufficient conditions may place demand on respiration for anabolic processes.

The lower compensation point of N fed plants in comparison with N starved plants is associated with very little change (on basis of

leaf area) or even a decrease (on basis of chlorophyll) in photosynthetic capacity. Thus the change in compensation point is likely to reflect disassimilatory processes.

Anaerobiosis has often been reported to inhibit photorespiratory activity (Ogren, 1984). In these experiments anaerobic conditions ($\text{CO}_2 + \text{N}_2$) resulted in marked reduction of disassimilation of CO_2 in normal N fed plants. Under such conditions oxygen is unavailable for the formation of glycolate during photorespiration (Ogren, 1984) and mitochondrial oxidation is inhibited. The residual disassimilatory CO_2 release may be due to anaerobic respiration or a result of photosynthetic net oxygen evolution causing partial relief from anaerobiosis.

Under anaerobic conditions the photosynthetic rate was significantly increased (Fig. 11a and Fig. 11b) indicating that photorespiration and respiration play a major rôle in determining net photosynthetic assimilation. This simplistic interpretation is open to question because anaerobiosis may stimulate photosynthetic CO_2 fixation in a number of ways apart from eliminating competition between O_2 and CO_2 for RuBPC/o. The anaerobic release of glycolytic control, mediated through the Pasteur effect, has as a consequence, increased metabolism of glucose under anaerobic conditions as a result of anaerobic metabolism. This would require the provision of increased amounts of photosynthate to the cytoplasm and thus a possible further enhancement of CO_2 assimilation. Anaerobic conditions may also reduce the magnitude of the Mehler reaction (pseudocyclic electron transport), which occurs under physiological conditions in higher plants (Gimmler, 1977), and which results in chloroplastic oxygen reduction to H_2O_2 thus releasing further reductant for CO_2 assimilation. Thus any changes in net photosynthetic activity should be viewed in the light of these possibilities. The mechanism through which N nutrition moderates photorespiration and/or respiration is not known although photorespiratory NH_3 cycling may play an important rôle (Ogren, 1984).

10.2. STOMATAL RESISTANCE

The possibility that some of the effects of inorganic nitrogen on photosynthetic carbon assimilation were the result of changes in stomatal resistance was investigated. The importance of stomatal resistance in determining photosynthetic rate was discussed by Farquhar and Sharkey (1982). Based on a model presented by these authors stomatal resistance only affects CO_2 assimilation at high resistances. Stomatal resistance has been shown to change diurnally keeping the ratio $(6A/5E)$ (A - assimilation rate, E - transpiration rate) constant at some optimal value (Hall and Schulze, 1980). Farquhar and Sharkey (1982) reported that phosphate in the transpirational stream promotes stomatal closure.

After removal of the roots from peas the stomatal resistance increased markedly (Fig. 16). This may be the result of a shock response by the plant as a result of the release of negative pressure in the xylem. When leaves of *Commelina communis* were excised (in air) from a well watered plant there was a transient increase in stomatal resistance (lasting 20 seconds) followed by a decrease in stomatal resistance over the next 100 seconds in turn followed by an increase in stomatal resistance (Martin et al., 1983a). These authors explain the transient increase as being the result of the release of tension in the continuous water columns of the xylem which results in the flooding of water into the mesophyll and epidermis so that the cells surrounding the guard cells have a temporary turgor advantage over the guard cells. This results in the closure of the guard cells. The subsequent changes in resistance in leaves excised in air is the result the guard cells equilibrating with the mesophyll and of the onset of water stress. The increase in stomatal resistance as a result of excision of the shoot under water found in this investigation may be analogous to the transient increase described by Martin et al. (1983a). This increase may have been prolonged as a result of cutting the shoots under water, thus preventing the onset of water stress. That the stomatal resistance recovered to nearly pre-cut levels over time indicates that transfer to de-aerated water did not affect this part of the physiology

extensively. The changes in stomatal resistance as a result of cutting would not have affected the results obtained for net photosynthetic CO_2 assimilation significantly because the plants were allowed to stabilize prior to introduction of ions into the bathing medium. These results do however explain why the photosynthetic rates took so long (1-2 hours) to stabilize.

Transfer of cut shoots into bathing media containing NO_3^- or NH_4^+ resulted in large concentration dependent effects (Fig. 17 and Fig. 18). Both 10 mM and 20 mM NaCl caused an increase in stomatal resistance. This may have been due to the effect of the salt on the water potential of the leaves, which has important consequences for stomatal apertures (Farquhar and Sharkey, 1982). In relation to the water control, 10 mM and 20 mM NO_3^- appeared to decrease stomatal resistance very slightly. In relation to the effect of similar concentrations of NaCl, NO_3^- decreased stomatal resistance. Ammonium increased stomatal resistance at both 10 mM and 20 mM concentrations above the level exhibited by the water control. At 10 mM NH_4^+ had less effect on stomatal resistance than NaCl while at the higher concentration (20 mM) NH_4^+ caused a huge increase in stomatal resistance.

Thus it may be concluded that both NO_3^- and NH_4^+ had some effect on stomatal resistance but that this was not the result of a 'salt' effect and that these effects were specific for the different ions. Similar results have been reported for Amaranthus powellii where stomatal resistance was reported to be negatively correlated with supply of NO_3^- up to 45 mM (Hunt et al., 1985c). These authors found that stomatal resistance and photosynthetic carbon assimilation rates were negatively correlated and speculated that both these physiological parameters are controlled by leaf nitrogen concentration or some factor correlated with leaf nitrogen. Farquhar and Sharkey (1982) report that stomatal resistance is positively dependent on pCO_2 . Thus increased photosynthetic rates which may result in decreased pCO_2 within the leaf or in the boundary layer may regulate stomatal aperture. This may thus result in stomatal resistance being partially controlled by photosynthetic rate.

The effects of NO_3^- and NH_4^+ on the stomatal resistance has important consequences for the interpretation of the results of photosynthetic carbon assimilation by intact plants. These effects of NO_3^- and NH_4^+ on stomatal resistance however were only observed at extremely high concentrations of the various salts. The lack of any evidence for such effects at lower concentrations may be due to the insensitivity of the technique used to the changes in stomatal resistance. It may be true that the photosynthetic measurements were more sensitive to stomatal resistance. The NO_3^- stimulation of photosynthetic activity may be partially attributable to decreased stomatal resistance although the changes in stomatal resistance with respect to the water control were not significant. The large inhibitory effects of NH_4^+ on photosynthetic carbon assimilation could however very well be attributed to changes in stomatal resistance.

10.3. LEAF DISK NET PHOTOSYNTHETIC CARBON DIOXIDE ASSIMILATION

Leaf disk net CO_2 assimilation was found to be influenced by the concentration and the time of NO_3^- infiltration. The rate of net $^{14}\text{CO}_2$ fixation by pea leaf disks declined with time for all treatments (Fig. 19). The disks were observed to dehydrate over time (10% loss in fresh mass over 30 minutes) and this may have led to a decline in photosynthetic activity. Ten minutes after infiltration of NO_3^- into the leaf disks the photosynthetic rate of disks treated with 2 mM NO_3^- exhibited greater net CO_2 assimilation than the controls and disks treated with more concentrated NO_3^- which caused some inhibitory effects. Similar results were observed for leaf disks after 20 minutes while after 30 minutes no inhibitory effects were noticed. Expressed differently; the time dependent reduction of net CO_2 assimilation was smaller in the treatments in which 0.5 mM and 2 mM NO_3^- were used than in the controls or the 0.2 mM treatment.

These results may be interpreted to mean that 0.2 mM NO_3^- has the greatest stimulatory effect on photosynthesis and that low concentrations of infiltrated NO_3^- are rapidly utilised and leaf

levels are depleted within 30 minutes. Thus nitrate at relatively high concentrations may partially reverse the time dependent decline of photosynthesis (see Fig. 19 control) although these high concentrations were initially weakly inhibitory on net CO_2 fixation. The forced entry of nitrate into the cells by vacuum infiltration prior to the labeling period means that any responses of the leaf disks are not due to uptake mechanisms into the leaf cells. The possibility of direct effects on the photosynthetic enzymes cannot be excluded. The time and concentration dependent effects of the NO_3^- correlate well with the hypothesis (discussed later) that the effects of NO_3^- on the photosynthetic rates are a consequence of its reduction requirements.

10.4. PROTOPLAST NITRATE, NITRITE AND AMMONIUM NET UPTAKE

If the metabolism of NO_3^- , NO_2^- and NH_4^+ is to be attributed effects on photosynthetic O_2 and CO_2 metabolism, it is pertinent to measure the rates at which these ions are utilised. If the ions are not metabolised to a significant extent the effects of the reduction requirements on photosynthesis are also likely to be insignificant.

10.4.1. Nitrate

No net uptake could be measured for NO_3^- by protoplasts *in vivo* probably due to a lack of sensitivity of the methods employed. Considering that the explanation of much of the experimental data is dependent on the presence of an active NR it was important to demonstrate the presence of this enzyme. Measurement of the accumulation of NO_2^- under dark anaerobic conditions allows the possibility of assessing the activity of this enzyme even although it does not provide any direct evidence for its function under normal physiological conditions. Increases in NR activity were observed even up to quite high concentrations of NO_3^- (10 mM) and thus it is possible to have reasonable confidence that the enzyme is active in the protoplast preparations.

10.4.2. Nitrite

Nitrite net uptake was found to occur at high rates within protoplasts. The method used for determination of NO_2^- net uptake involved the incubation of intact protoplasts in NO_2^- containing medium prior to the breakage of the protoplasts and the determination of NO_2^- loss from the whole system. Thus NO_2^- had to be taken up into the protoplast prior to reduction within the chloroplast. Uptake of NO_2^- has been reported to occur across the plasmalemma and the chloroplast envelope (Kaiser and Heber, 1983) although the latter membrane system was considerably more permeable to the ions (200 fold). It has also been reported that NO_2^- is taken up predominantly in its protonated form (Kaiser and Heber, 1983). Protonation occurs chemically in a pH dependent fashion with an equilibrium lying to the left of the reaction ($\text{NO}_2^- + \text{H}^+ \longrightarrow \text{HNO}_2$).

Whatever the effects of NO_2^- on photosynthetic net O_2 evolution or net CO_2 fixation, the net uptake/reduction of NO_2^- occurred at high rates ($1.1 \mu\text{moles} \cdot \text{mg chl}^{-1} \cdot \text{h}^{-1}$). The rate of NO_2^- net uptake was concentration dependent and increased rapidly between 0 and 0.2 mM NO_2^- (Fig. 21 and Fig. 22). At concentrations greater than 0.2 mM the rate of net uptake was reduced. The pattern of NO_2^- net uptake in response to NO_2^- is reminiscent of enzymatic kinetics. Considering the absence of any evidence for active uptake of NO_2^- across either the plasmalemma or the chloroplast envelope, it is likely that the influx of NO_2^- into the cell is reduction related. It may be speculated that the decline in net uptake rate at high concentrations may be due to the accumulation of the reduction products of NO_2^- in the chloroplast.

10.4.3. Ammonium

Efflux of ammonium from cells treated with extremely high concentrations of NH_4^+ occurred within 8 minutes of initiation of NH_4^+ supply (Fig. 24). At lower concentrations the net uptake

continued over time although a decrease in the rate of net uptake was observed after 20 minutes at 1.5 mM NH_4^+ . This decrease must have been associated with an efflux of previously accumulated NH_4^+ . The efflux of NH_4^+ may have resulted from an over loading of cellular pools and a breakdown in the ability of the membranes to retain absorbed ammonium or an active excretion of NH_4^+ .

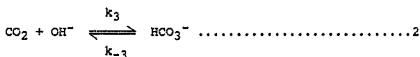
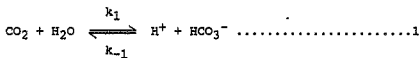
The net uptake of NH_4^+ showed a response to exogenous levels reminiscent of enzymatic responses to substrate, but this pattern was disrupted by a decline in quantity taken up at higher concentrations with efflux occurring at the highest concentration (Fig. 25. and Fig. 26). The decline in net uptake may have been the net result of an increase in efflux in comparison to influx as suggested by the net release of NH_4^+ at 2 mM.

10.5. PROTOPLAST NET CARBON DIOXIDE ASSIMILATION

The use of protoplasts provided a number of advantages over other tissue systems. The protoplasts were directly exposed to the ionic treatments at the plasmalemma level and thus vagaries of the uptake and transport systems were eliminated. The question of the validity of the use of protoplasts as physiological tools arises from the osmotic stress encountered by protoplasts, transfer into a new solute environment, removal of the cell wall and breakage of plasmodesmata (Robinson and Loveys, 1986). Changes in membrane composition during isolation and incubation have recently been reported not to occur (Goldberg et al., 1986). Sugar is normally used as an osmoticum for protoplast preparation and serves to plasmolyse the cells and provide osmotic support. Plasmolysis aids digestion and breakage of plasmodesmata but also has other physiological effects on protein synthesis, photosynthesis, respiration, solute uptake, electrolyte efflux and membrane potentials (Robinson and Loveys, 1986). The effects of the osmotica used has recently been discussed by these authors who found that the disaccharide sugar alcohols used commonly (sorbitol and mannitol), and also sucrose, do penetrate the

cells and are uniformly distributed within the cytoplasm.

There is some discussion in the literature over the form of diffusible inorganic carbon (DIC) which is favoured for uptake across biological membranes. Lipid bilayers are far more permeable to CO_2 than to HCO_3^- and passive flux of HCO_3^- is therefore expected to be minimal. (Espie and Colman, 1986). Thus the effectiveness of the supply of HCO_3^- is dependent on the conversion of HCO_3^- to CO_2 . The following equations (from Espie and Colman, 1986) describe this conversion:



The equilibrium constant (K_{a1}) of equation 1 is given by:

$$K_{a1} = \frac{k_1}{k_{-1}} = \frac{[\text{H}^+][\text{HCO}_3^-]}{[\text{CO}_2]}$$

and

$$\frac{K_{a1}}{K_w} = \frac{k_3}{k_{-3}} = \frac{[\text{HCO}_3^-]}{[\text{CO}_2][\text{OH}^-]}$$

where $K_w = [\text{H}^+][\text{OH}^-]$

The following values are derived from Espie and Colman, 1986)

$$K_{a1} = 4.45 \times 10^{-7} \text{ M}$$

$$K_w = 1.01 \times 10^{-14} \text{ M}$$

Using these values with the HCO_3^- concentration at 10 mM, the concentration of CO_2 in the solution at 25°C was $5.8 \times 10^{-4} \text{ M}$ (0.58 mM). Thus there are significant levels of CO_2 available at the pH and HCO_3^- concentration of the buffers used. Water at 25°C

becomes saturated at 0.145 g/100 ml CO_2 ie. 33 mM (CRC Handbook of Physics and Chemistry, 1982) at a pressure of $1.013 \times 10^5 \text{ N.m}^{-2}$. Under these conditions and normal atmospheric CO_2 , water contains 0.011 mM CO_2 of unspecified form (Golterman, 1969) in comparison to 10 mM CO_2 of an unspecified form when NaHCO_3 was added. This analysis allows us to conclude that the provision of NaHCO_3 elevates the CO_2 levels beyond those normally experienced by the plant and thus photorespiration may be assumed to be negligible.

10.5.1. Nitrate

A biphasic response pattern was observed for the effects NO_3^- on protoplast net $^{14}\text{CO}_2$ assimilation (Fig. 27). Typical raw data are graphed in Fig. 29 and show that the responses to NO_3^- occurred within seconds of application. Thus synthetic mechanisms may be eliminated from our considerations. The compression of the response pattern into a narrower concentration range at low light intensities (Fig. 29) provides some evidence for the lack of any direct effects on photosynthetic enzyme activities. If NO_3^- was able to directly activate the photosynthetic enzymes the degree of the response may have been light dependent, but the concentration range over which the effects would occur should have remained similar.

These data may be interpreted to fit with an hypothesis on the origin of reductant for NO_3^- reduction and the effects that the utilization of this reductant may have on photosynthetic carbon assimilation. The reduction of NO_3^- is thought to be a cytoplasmic event and some dependence on photosynthesis has been suggested (Naik et al., 1982; Calvin and Atkins, 1974). This dependence may be direct or indirect, but the ultimate source of reduction potential is the chloroplast. Direct effects may be grouped as those which involve an export of some products from the chloroplast to the cytoplasm which are then immediately available for participation in NO_3^- reduction. Indirect dependence stems from the reliance of respiration on photosynthesis for provision of carbon substrates.

During active photosynthesis TP export is the major drain of carbon from photosynthesis (Stitt and Heldt, 1985). This TP may be diverted into starch synthesis within the chloroplast by the absence of cytoplasmic Pi which normally exchanges across the chloroplast membrane for TP (Heldt and Rapley, 1970). In addition the control of sucrose synthesis by the metabolite fructose 2,6-bisphosphate and the re-release of Pi during sucrose synthesis are thought to be important for control of cytoplasmic Pi levels (Champigny, 1985). Nitrate has been shown to regulate the accumulation of starch within the chloroplast (Ariovich and Cresswell, 1983; Kerr et al., 1984; Huber et al., 1985). The influence of NO_3^- on these processes may be due to two mechanisms:

- a) NO_3^- has been suggested to regulate the ATPase dependent vacuolar storage of Pi (Lamaze et al., 1984)
- b) NO_3^- may increase the rate of TP utilization thus preventing starch accumulation.

The export of TP has been suggested to be coupled to NO_3^- reduction through the glycolytic metabolism of DHAP to PGA with the concomitant reduction of NAD to NADH (Klepper et al., 1971). The PGA thus produced has two possible fates. It may be retained in the cytoplasm, or returned into the chloroplast in exchange for further DHAP. These two possibilities have distinct consequences for carbon assimilation. Other possibilities include the operation of the malate-oxaloacetate shunt and a glucose translocator (Heber and Heldt, 1981). The former results in the export of light reaction reducing potential to the cytoplasm and there is very little commentary on the latter in the literature.

Thus the results of the current investigation may be the consequence of the control of cytoplasmic Pi concentrations by NO_3^- and thus the regulation of photosynthesis through control of TP export. This mechanism has similar consequences to the control of TP export through the reduction requirements of NO_3^- . The supply of NO_3^- may increase the export of DHAP or malate from the chloroplast for the provision of reducing equivalents. If malate were exported in a shuttle system the reducing potential would be

derived from the light reaction competitively with the Calvin cycle. The result of this could only be an inhibition of photosynthetic carbon assimilation by NO_3^- . Work done by Rathnam (1978) using inhibitors of the Calvin cycle suggested that DHAP was the physiological source of reductant in spinach. If NO_3^- stimulates DHAP export from the chloroplast a stimulation of photosynthetic carbon assimilation may be anticipated as a result of a shift in the balance of the photosynthetic mass-action equation. The increased export of TP would result in a decrease in starch accumulation. Starch accumulation is thought to disrupt chloroplastic structure and function (Ariovich and Cresswell, 1983; Kerr et al., 1984) and thus a lack of accumulation may have beneficial consequences. These effects are fundamentally the result of a removal of feedback inhibition through the elimination of carbon from the chloroplast. This is only a consequence of DHAP export if PGA is not returned to the chloroplast, but further metabolised by cytoplasmic pathways. In such a situation DHAP may be exchanged for Pi across the chloroplast membrane. If PGA is shuttled back into the chloroplast only reducing potential is exported via this cycle.

This reasoning may explain the stimulatory effects of NO_3^- on net photosynthetic carbon assimilation at high light intensities and low nitrate concentrations. Under these conditions the Calvin cycle would be operating maximally and export of TP may not deplete chloroplastic metabolites significantly. If the light intensity is reduced the ongoing export of carbon for NO_3^- reduction may result in a depletion of metabolites required for the functioning of the Calvin cycle (Ru5P) (Stitt and Heldt, 1985). A similar effect may be induced by high NO_3^- concentrations. An additional possibility is that at high NO_3^- concentrations or low light intensities the dicarboxylate and/or DHAP/PGA shunts come into operation resulting in NO_3^- reduction being competitive with CO_2 assimilation. These pathways may play some rôle in any case. Their existence is well known and if these shuttles are operating it is likely that at least some of the reductant exported will be utilised for NO_3^- reduction. The major pathway may be TP export with extra reductant supplied from the shuttles.

It may be argued that the results of the addition of NO_3^- are merely the result of the effects of the reduction products of NO_3^- on net CO_2 fixation. That this is the case seems unlikely in view of the strong inhibitory effects of NO_2^- and NH_4^+ on net CO_2 assimilation. It is possible that the inhibitory effects of NO_3^- at high concentrations result from the effects of its reduction products.

10.5.2. Nitrite

Nitrite inhibited $^{14}\text{CO}_2$ assimilation by protoplasts (Fig.30). Nitrite reduction is light reaction dependent and thus may compete with the Calvin cycle for reductant. Evidence for such competition has been derived from CO_2 suppression of NO_2^- reduction (Larsson et al., 1985) and stimulation of NO_2^- reduction by inhibitors of the Calvin cycle (Anderson and Done, 1985). In contrast NO_2^- has been reported to stimulate CO_2 assimilation (Robinson, 1986). Calculations of $\text{Fd}_{(\text{rd})}$ content of leaf tissue led to the conclusion that there is sufficient $\text{Fd}_{(\text{rd})}$ for both NO_2^- and CO_2 assimilation without competition (Buchanan, 1980; Robinson, 1983).

The most common report in the literature is that NO_2^- inhibits net CO_2 fixation (Larsson et al., 1985; Purczeldt et al., 1978). This effect may be due to stromal acidification (Purczeldt et al., 1978; Larsson et al., 1985) resulting in inhibition of the key enzymes (fructose and sedoheptulose biphosphatase) of carbon assimilation (Purczeldt et al., 1978; Kaiser and Heber, 1983). Nitrite is thought to freely permeate the chloroplast membrane, predominantly in the form of HNO_2 (Kaiser and Heber, 1983) and thus transport is not likely to be energy dependent.

Although NO_2^- may or may not intercompete for Fd with carbon assimilation, NO_2^- does appear to inhibit carbon assimilation through its chemical effects on the stroma. The effects of NO_2^- may be partially the result of NH_4^+ effects, as the two ions resulted in very similar responses of net CO_2 assimilation.

10.5.3. Ammonium

Ammonium resulted in a very transient small increase in net photosynthetic $^{14}\text{CO}_2$ assimilation at low (0.033 mM) NH_4^+ concentration (Fig. 31). Assimilation of NH_4^+ is light dependent and requires the presence of $\text{Fd}(\text{rd})$ and ATP (Klaus et al., 1985). Supply of NH_4^+ has been reported to increase the incorporation of $^{14}\text{CO}_2$ into amino acids and decrease incorporation into starch and sucrose (Platt et al., 1977). These changes were accompanied by an increase in pyruvate and a decrease in PEP. Pyruvate may provide the carbon skeletons for glutamate and glutamine for the assimilation of NH_4^+ into amino acids. Lea and Miflin (1979) found little evidence to suggest that NH_4^+ incorporation into amino acids functioned with the direct participation of carbon from photosynthesis. It was suggested to be more likely that TP exported from the chloroplast was re-imported as α -keto acids. Other workers report that freshly assimilated CO_2 is utilised from organic acids and carbohydrates (Atkins and Calvin, 1975; Klaus et al., 1985). Thus NH_4^+ assimilation would be indirectly dependent on the Calvin cycle for carbon.

Ammonium assimilation is undoubtedly linked to the light reaction for the provision of $\text{Fd}(\text{rd})$ (Atkins and Calvin, 1975; Larsson et al., 1985). Thus the assimilation of ammonium may be expected to compete with CO_2 fixation for reductant. In addition ammonium is known to uncouple photophosphorylation through its protonophore effect and high concentrations inhibit the light reaction (Izawa, 1977).

The initial stimulation of net $^{14}\text{CO}_2$ assimilation may result from the utilization of photosynthetic carbon for amino acid synthesis, while inhibition was a consequence of competition for $\text{Fd}(\text{rd})$ and uncoupling/inhibitory effects. The initially enhanced photosynthetic rate may function in the regulation of photorespiration by reducing the ratio of CO_2/O_2 in the leaf thus facilitating increased photorespiration, although such mechanisms are unlikely to function in the system (protoplasts) used for these studies as a result of the elevated CO_2 levels.

10.6. PROTOPLAST NET OXYGEN EVOLUTION

As a result of the interdependence of photosynthetic O_2 evolution and CO_2 assimilation, any factor influencing the operation of one of these pathways influences the other. Thus many of the effects of inorganic nitrogen on net O_2 evolution may be traced to effects on CO_2 fixation.

10.6.1. Nitrate

Comparison of the stimulatory effects of NO_3^- on net CO_2 fixation (Fig. 27a) and net O_2 evolution (Fig. 33) shows that NO_3^- has a much greater effect on the latter. The magnitude of the response may be a result of the demands placed on the light reaction for the further metabolism of NO_3^- reduction products. Nitrate has been reported to influence the activity of the water-oxidation step of the light reaction (Osman, 1982). Nitrate has already been suggested to play a rôle in the stimulation of carbon assimilation which would increase the demand for the products of the light reaction. Nitrate reduction may be at least partially linked to TP exported and thus dependent on the operation of either the PGA/DHAP or dicarboxylate shuttles.

The decline in the stimulatory effect of NO_3^- at higher concentrations may be due to its effects on CO_2 assimilation, ie. through the inhibition of net CO_2 assimilation at high NO_3^- concentrations.

10.6.2. Nitrite

Nitrite stimulated net photosynthetic O_2 evolution over a wide range of concentrations (Fig. 34). This is in contrast to the inhibitory effects of NO_2^- on CO_2 . This increase occurred in spite of the decrease in the demand for reductant by the Calvin cycle during incubation with NO_2^- . It is possible that the reduction of

NO_2^- was responsible for the enhanced net O_2 evolution. Similar results have been reported by other workers (Furczeldt et al., 1978) who found both an enhancement of net O_2 evolution, which was bicarbonate dependent, and a decrease in net CO_2 assimilation as a result of stromal acidification.

The high concentration decline in NO_2^- stimulation of net O_2 evolution may be attributable to strong acidification of the stroma.

10.6.3. Ammonium

Ammonium caused a significant elevation (10%) of net O_2 evolution at low concentrations (Fig. 35). The response of net O_2 evolution essentially paralleled that of net CO_2 fixation. The initial stimulation of net O_2 evolution by ammonium may suggest an uncoupling effect, although if this was associated with the stimulation of net CO_2 fixation, uncoupling is unlikely. Comparison of net O_2 evolution and net CO_2 assimilation (Fig. 31 and Fig. 35) suggests that the effect of NH_4^+ on net O_2 evolution may be a result of a combination of these effects. The concentration at which NH_4^+ became inhibitory on net O_2 evolution was much higher than the analogous concentration for net CO_2 fixation. Thus it is possible that stimulation of CO_2 assimilation contributed partially to elevated net O_2 evolution, but that the uncoupling effect described by Izawa (1977) was a major contributor. Ammonium is known to uncouple photophosphorylation at low concentrations and have an inhibitory effect on the light reaction at higher concentrations (Izawa, 1977). The alternative possibility is that the demand for reductant from the light reaction stimulated net O_2 evolution.

10.6.4. Phosphate

The rôle of phosphate in transport of TP out of the chloroplast has been discussed previously. The importance of this ion in the metabolism of carbon was probably the reason for the stimulatory

effect of PO_4^{2-} on net O_2 evolution (Fig. 36). At low concentrations phosphate is likely to stimulate photosynthetic net O_2 evolution as a result of increased carbon metabolism resulting from improved TP export from the chloroplast. At higher concentrations of PO_4^{2-} , the withdrawal of large amounts of TP may deplete chloroplastic intermediates from the Calvin cycle and thus inhibit CO_2 fixation. In addition PO_4^{2-} has been reported to stimulate the activity of the Hill reaction (Sawada et al., 1984).

10.6.5. Sodium bicarbonate

The presence of a carbon dioxide source in the incubation medium of protoplasts is likely to be of considerable importance for the functioning of the protoplasts in terms of the CO_2 requirements of photosynthesis. In these experiments, CO_2 was supplied in the form of NaHCO_3 , which should dissociate to CO_2 and HCO_3^- to a small but significant extent at the pH of the incubating medium (pH 7.6). In all experiments bicarbonate was supplied at 10 mM which should saturate photosynthesis. Supply of different amounts of bicarbonate was found to dramatically increase net O_2 evolution (Fig. 37). The response to bicarbonate was positive for all concentrations supplied. Without added bicarbonate net O_2 evolution did not occur or occurred at a low rate. This was in spite of the de-aeration of the medium prior to the experiment. Thus it appears that there may be a CO_2 independent component of net O_2 evolution although it is not certain that the medium was completely depleted of CO_2 and photorespiratory and respiratory CO_2 release may play a rôle in maintaining a minimal rate of photosynthetic net O_2 evolution. In addition the photosynthetic system may be supplying reductant and ATP to many processes within the cells and it is therefore not surprising that net O_2 evolution should occur in the absence, even if only partial, of CO_2 . The pre-incubation treatment of the protoplasts was significant in this regard. In protoplast preparations pre-incubated in the light no initial (prior to addition of NaHCO_3), or very little, O_2 evolution occurred. Protoplasts incubated in the dark showed greater initial rates of O_2 evolution. It must be remem-

bered that any evolution of O_2 is the net flux resulting from photosynthetic O_2 production, respiratory and photorespiratory O_2 reduction.

With the addition of bicarbonate to the medium net O_2 evolution was almost linear with NaH_2CO_3 concentration, indicating the strict interdependence of these two processes. High concentrations of HCO_3^- (30 mM) have been reported to result in stromal acidification (Kaiser and Heber, 1983) and this may have been the reason for the decline in the stimulatory effect of bicarbonate at concentrations above 15 mM. The presence of CO_2 in the incubation medium has other important ramifications. Elevated CO_2 levels may depress photorespiratory O_2 consumption by increasing the competitive potential of CO_2 for RuBPC. In addition the Mehler reaction (pseudo-cyclic reduction of oxygen to H_2O_2) is favoured under conditions where CO_2 fixation is inhibited (Radmer and Kok, 1976) and thus increased CO_2 should reduce the contribution of this reaction to net O_2 consumption.

10.6.5.1. Interaction between Nitrate and Sodium Bicarbonate

A significant finding was that NO_3^- has no, or very little, stimulatory effect on the net evolution of O_2 by protoplasts in the absence of $NaHCO_3$. This is especially striking if it is noted that the initial rates of net O_2 evolution by protoplasts not supplied with $NaHCO_3$ were equivalent to those of protoplasts supplied with 1 mM $NaHCO_3$ and yet the responses of these two treatments to NO_3^- were markedly different. The trend appears to be that with higher $NaHCO_3$ concentrations in the medium, greater stimulation of net O_2 evolution by NO_3^- is possible and at higher concentrations. In addition the net evolution of O_2 appears to be more resistant to higher, inhibitory, levels of NO_3^- under conditions of greater $NaHCO_3$ supply.

These results may imply that CO_2 is required for the stimulatory effect of NO_3^- on net O_2 evolution and lend support to the hypothesis that the effect of NO_3^- on net O_2 evolution is through the reduction requirements of NO_3^- for carbon exported from the

chloroplast. At low NO_3^- concentrations (1 mM) no inhibition of net O_2 evolution was observed. This may be suggested to mean that at these concentrations (1mM), the effect of carbon withdrawal from the Calvin cycle was not enough to significantly deplete the pools of intermediates although this depletion was sufficient to stimulate net O_2 evolution. At higher concentrations of NO_3^- , especially under conditions of low CO_2 , the removal of carbon intermediates from the Calvin cycle was significant enough to cause a breakdown in this cycle. The fact that 1 mM NaHCO_3 was found to reverse the inhibitory effects of high NO_3^- concentrations on protoplasts incubated without NaHCO_3 is significant in that it indicates that NO_3^- induced suppression of photosynthetic net O_2 evolution is a result of a carbon limitation. It is possible that NO_3^- may reduce respiratory O_2 consumption (Frank and Marek, 1983). In experiments conducted in the dark it was impossible to discern any effect of NO_3^- on net O_2 consumption. The fact that increased responses to NO_3^- were observed at higher NaHCO_3 concentration precludes the possibility of photorespiration playing a rôle in these responses.

10.6.5.2. Interaction between Ammonium and Sodium Bicarbonate

During the metabolism of NH_4^+ to amino acids carbon skeletons are required. It is thus commonly supposed that NH_4^+ should place additional demands on the Calvin cycle for carbon. In these experiments it appeared that the stimulatory effect of NH_4^+ on net O_2 evolution declined with increasing NaHCO_3 concentration. It would thus appear that the effects of NH_4^+ on net O_2 evolution are at least partially independent of CO_2 metabolism. Alternatively it may be possible that NH_4^+ has the ability to increase net O_2 evolution only to a certain extent and that the supply of NaHCO_3 masked this effect. A more tenable hypothesis is that the reduction requirements of NH_4^+ compete with CO_2 for reductant and that, under low CO_2 conditions, NH_4^+ is a fairly successful competitor, but not at higher CO_2 concentrations.

The low concentration NH_4^+ uncoupling of photophosphorylation and higher concentration inhibition of photosynthetic electron

transport have been previously discussed. It is possible that these factors may play a rôle here especially at the higher concentrations where NH_4^+ was found to inhibit net O_2 evolution. It is perhaps noteworthy that NaHCO_3 was unable to reverse NH_4^+ inhibition of net O_2 evolution, indicating the lack of a strong direct link between the two processes.

10.6.5.3 The effects of respiratory inhibitors

In order to better elucidate the rôle played by respiration in the responses discussed above, MFA was used to inhibit respiration. The effectiveness and specificity of this inhibitor is questionable, but it at least partially inhibits respiration (Elrifí and Turpin, 1986) and appears to leave photosynthetic O_2 evolution intact. In a measurement of respiratory O_2 evolution (under dark conditions) very little O_2 consumption was observed and this declined to no consumption at all within a few minutes.

Supply of MFA stimulated net O_2 evolution above the rates observed for protoplasts not exposed to MFA, regardless of the concentration of NaHCO_3 supplied. This may result from the partial depression of respiratory O_2 consumption. The greater stimulation of net O_2 evolution by MFA incubated cells in contrast to normal cells in the presence of 1 mM NO_3^- and the lack of inhibition by an additional 3 mM may also be ascribed to the partial lack of respiratory O_2 consumption acting as a buffer to changes in net O_2 evolution.

Cyanide as an inhibitor of respiration provides a special and interesting case. Cyanide inhibits electron transport in both photosynthesis and respiration. It may thus be surprising that the levels of O_2 changed at all after the addition of cyanide. The Mehler reaction has been reported to be insensitive to cyanide (Gimmler, 1977) and Radmer and Kok (1976) reported high rates of O_2 uptake by algae after the supply of cyanide. During normal photosynthesis O_2 evolution is far in excess of O_2 fixation by the Mehler reaction which inhibits non-cyclic electron transport, inhibits reduction and thus CO_2

fixation. The electrons from pseudocyclic electron transport are then available for the reduction of O_2 and thus O_2 is taken up in the light in excess of O_2 evolution. This process is dependent on presence of light and this may explain why, when the protoplasts were transferred to dark conditions, the rate of net O_2 consumption decreased and partially resumed on return to light conditions.

10.7. EFFECT OF NITRATE ON PROTOPLAST ATP LEVELS

The effects of NO_3^- on the accumulation of ATP in the protoplasts are difficult to interpret as a result of the large number of sites for ATP synthesis and ATP hydrolysis. The levels of ATP measured reflect a disequilibrium situation between the production and the utilization of ATP. The initial decrease in the levels of ATP on supply of NO_3^- (Fig. 47) may result from the requirement for the products of the dark reaction of photosynthesis (DHAP) by NO_3^- reduction and the increased synthesis of these metabolites. The reduction of NO_3^- in the cytoplasm, mediated by the reduction of NAD by DHAP to yield NADH and PGA , is accompanied by a release of ATP. The accumulation of ATP after an initial depletion (Fig. 48) may reflect the operation of this pathway. Ascribing the accumulation of ATP to any one reaction is however a dangerous assumption. The accumulation may also be the result of increased mitochondrial activity as a result of depletion of cytoplasmic DHAP.

11. CONCLUSION

The short term and long term responses of photosynthesis to inorganic nitrogen are the result of different mechanisms. The long term effects of N nutrition may result in elevated protein synthesis, photosynthesis and growth (Hunt et al., 1985c) although specific increases in the levels of several enzymes are also possible (Tew, 1976). Stomatal resistance may play an important rôle in determining the effects of particularly ammonium on net photosynthesis. Supply of nitrogen causes a decrease in the compensation point. Changes in photorespiratory activity may play an important rôle in determining the rate of net photosynthesis. These effects are not dependent on the continual presence of N in the growth medium.

The predominant short term effect of inorganic nitrogen on photosynthetic carbon metabolism may be speculated to be mediated through the requirements of these ions for reductant and carbon during their assimilation into amino acids. This class of effects is dependent on the presence of the inorganic nitrogen within the cell.

The mechanisms through which the various inorganic nitrogen species influence carbon metabolism may be hypothesized to be the following.

Nitrate reduction is cytoplasmic and is supplied with reductant from the export of H_2 from the Calvin cycle. This carbon is metabolised via the glycolytic sequence to yield PGA, NADPH and ATP. The PGA may be further metabolised through the respiratory pathways or some returned to the chloroplast in exchange for further DHAP. The dicarboxylate shunt may also operate to some extent in the provision of

reductant. Nitrate may also exert a direct effect on the light reaction.

Nitrite reduction is chloroplastic and utilizes $Fd_{(rd)}$ produced by the light reaction. Nitrite may or may not compete with the light reaction for reductant. Nitrite influx into the chloroplast results in the acidification of the stroma which inhibits CO_2 fixation.

Ammonium utilizes chloroplastic $Fd_{(rd)}$ and ATP for its assimilation. This utilization of reductant may or may not compete with the Calvin cycle. The carbon utilized for NH_4^+ assimilation into amino acids may be derived from concurrent photosynthesis. Exogenous supply of NH_4^+ causes uncoupling of photophosphorylation and inhibition of the light reaction at low and high concentrations respectively.

The postulates pertaining to the effects of the various inorganic nitrogen ions on net photosynthetic carbon assimilation are described in the form of a metabolic scheme in Fig. 48. The presence of inorganic nitrogen in the form of NO_3^- is concluded to be of importance to photosynthetic carbon metabolism. Without adequate NO_3^- export of TP from the chloroplast is likely to be impaired with the resulting deleterious accumulation of starch within the chloroplast. Maximal productivity can be expected in plants supplied with a continuous source of NO_3^- rather than large infrequent applications. Nitrite appears not to have beneficial effects on carbon assimilation while NH_4^+ may be effective in mediating some positive effects on photosynthesis.

12. REFERENCES

- Amory, A.M. and Cresswell, C.F. (1971). In Vitro effects of inorganic nitrogen ions on ribulose-1, 5-bisphosphate carboxylase/oxygenase activities of Themeda triandra and Zea mays. Unpublished.
- Amory, A. and Cresswell, C.F. (1984). Effect of inorganic nitrogen ions on photosynthesis and carbon dioxide compensation concentration of Themeda triandra and Zea mays. Ann. Bot., 54: 719-727.
- Amory, A. and Cresswell, C.F. (1986). Role of formate in the photorespiratory metabolism of Themeda triandra Forssk. J. Plant Physiol., 124: 247-255.
- Anderson, J.W. (1981). Light-energy-dependent processes other than CO₂ assimilation. In: The Biochemistry of Plants, Vol. 8., Ed Hatch, M.D. and Boardman, N.K., pp. 473-500, Academic Press, New York, London.
- Anderson, J.W. and Done, J. (1981). Light-dependent assimilation of nitrite by isolated pea chloroplasts. Plant Physiol., 61: 692-697.
- Anderson, J.W. and House, C.M. (1979). Polarographic study of oxaloacetate reduction by isolated pea chloroplasts. Plant Physiol., 64: 1058-1063.
- Anderson, J.W. and Walker, D.A. (1983). Ammonia assimilation and oxygen evolution by a reconstituted chloroplast system in the presence of 2-oxoglutarate and glutamate. Planta, 159: 247-253.
- Andersson, B., Felle, H. and Benstrup, F. (1984). Amine transport in Riccia fluitans. Plant Physiol., 76: 75-78.
- Aparicio, P.J. and Maldonado, J.M. (1977). Regulation of nitrate assimilation in photosynthetic organisms. In: Sixth Long Ashton Symposium Ed. Hewitt, E.J. and Cutting, C.V. pp. 153-163. Academic Press, New York, London.
- Ariovich, D. and Cresswell, C.F. (1983). The effect of nitrogen and phosphorus on starch accumulation and net photosynthesis in two variants of Panicum maximum Jacq. Plant, Cell and Environment: 6-657.
- Arnon, D.I. (1949). Copper enzymes in isolated chloroplasts. Polyphenoloxidase in Beta vulgaris. Plant Physiol., 24: 1-15.
- Ashley, D.A., Jackson, W.A. and Volk, R. (1975). Nitrate uptake and assimilation by wheat seedlings during initial exposure to nitrate. Plant Physiol., 55: 1102-1106.
- Aslam, M. (1981). Re-evaluation on anaerobic nitrite production as an index for the measurement of metabolic pool of nitrate. Plant Physiol., 68: 305-308.
- Aslam, M. and Huffacker, R.C. (1982). In vivo nitrate reduction in roots and shoots of barley (Hordeum vulgare L.) seedlings in light and darkness. Plant Physiol., 70: 109-1013.

Aslam, M., Oaks, A. and Huffacker, R.C. (1976). Effect of light and glucose on the induction of nitrate reductase and on distribution of nitrate in etiolated barley leaves. *Plant Physiol.*, 58: 588-591.

Atkins, C.A. and Canvin, D.T. (1975). Nitrate, nitrite and ammonium assimilation by leaves: Effects of inhibitors. *Planta*, 123: 41-51.

Avdeeva, T.A. and Andreeva, T.F. (1973). Nitrogen nutrition and activities of CO₂-fixing enzymes and glyceraldehyde phosphata dehydrogenase in broad bean and maize. *Photosynthetica*, 7: 140-145.

Barskii, E.L., Gusev, M.V., Kazennova, N.V. and Samuilov, V.D. (1985). Inhibiting action of uncouplers on photosynthetic oxygen evolution by cells of the cyanobacterium Anabaena variabilis. *Soviet Plant Physiol.*, 32: 526-531.

Baskakova, S.Y.U. and Izmailov, S.Z. (1985). Regulation of glutamine synthetase and glutamate dehydrogenase activities during heterotrophic and nitrate nutrition of plants. *Soviet Plant Physiol.*, 31: 870-875.

Baysdorfer, C. and Robinson, J.M. (1985). Metabolic interactions between spinach leaf nitrite reductase and ferredoxin-NADP reductase. *Plant Physiol.*, 77: 318-320.

Ben-Shalom, N., Huffacker, R.C. and Rappaport, L. (1983). Effect of photosynthetic inhibitors and uncouplers of oxidative phosphorylation on nitrate and nitrite reduction in barley leaves. *Plant Physiol.*, 71: 63-66.

Ben-Zioni, A., Vaadia, Y. and Lips, S.H. (1971). Nitrate uptake by roots as regulated by nitrate reduction products of the shoot. *Physiol. Plant.*, 24: 288-290.

Berger, M.G. and Fock, H.P. (1983). Effects of methionine sulfoximine and glycine metabolism of maize leaves in the light. *Aust. J. Plant Physiol.*, 10: 187-194.

Berger, M.G. and Fock, H.P. (1985). Comparative studies on the photorespiratory nitrogen metabolism in wheat and maize leaves. *J. Plant Physiol.*, 119: 257-267.

Bergmann, L., Grosse, W. and Koth, P. (1976). Influences of ammonium and nitrate on N-metabolism, malate accumulation and malic enzyme activity in suspension cultures of Nicotiana tabacum. *Z. Pflanzenphysiol.*, 80: 60-70.

Bertl, A., Felle, H. and Bentrup, F. (1984). Amine transport in Riccia fluitans. *Plant Physiol.*, 76: 75-78.

Bidwell, R.G.S. (1974). *Plant Physiology*. Macmillan, New York.

Blackwood, G.C. and Miflin, B.J. (1976). The effect of nitrate and ammonium feeding on carbon dioxide assimilation in maize. *J. Experimental Bot.*, 27: 735-747.

Blom-Zandstra, M. and Lampe, J.E.M. (1985). The role of nitrate in the osmoregulation of lettuce (Lactuca sativa L.) grown at different light intensities. *J. of Experimental Bot.*, 36: 1043-1052.

Bloom, A. and Finazzo, J. (1986). The influence of ammonium and chloride on potassium and nitrate absorption by barley roots depends on time of exposure and cultivar. *Plant Physiol.*, 81: 67-69.

- Boudet, A.M., Alibert, G. and Marigo, G. (1984). Vacuoles and tonoplast in the regulation of cellular metabolism. pp. 29-47.
- Bretler, H. and Luczak, W. (1982). Utilization of nitrite and nitrate by dwarf bean. *Planta*, 156: 226-232.
- Buchanan, B.B. (1980). Role of light in the regulation of chloroplast enzymes. *Ann. Rev. Plant Physiol.*, 31: 341-374.
- Butz, R.G. and Jackson, W.A. (1977). A mechanism for nitrate transport and reduction. *Biochem.*, 15: 409-417.
- Canvin, D.T. and Atkins, C.A. (1974). Nitrate, nitrite and ammonium assimilation by leaves: Effect of light, carbon dioxide and oxygen. *Planta*, 116: 207-224.
- Canvin, D.T. and Woo, K.C. (1979). The regulation of nitrate reduction in spinach leaves. *Can. J. Bot.*, 57: 1155-1160.
- Cataldo, D.A., Haroon, M., Schrader, L.E. and Youngs, V.L. (1975). Rapid colorimetric determination on nitrate in plant tissue by nitration of salicylic acid. *Commun. Soil Science and Plant Analysis*, 6: 71-80.
- Chmipigny, M.L. (1985). Regulation of photosynthetic carbon assimilation at the cellular level: a review. *Photosynthesis Research*, 6: 273-286.
- Chaparro, A., Maldonado, J. M., Diez, J., Reliempio, A.M., and Loasda, M. (1976). Nitrate reductase inactivation and reducing power and energy charge in *Chlorella* cells. *Plant Science Letters*, 6: 335-342.
- Clarkson, D.T., Hopper, M.J. and Jones, H.P. (1986). The effect of root temperature on the uptake of nitrogen and the relative size of the root system in *Lolium perenne*. I. Solutions containing both NH_4^+ and NO_3^- . *Plant, Cell and Environment*: 9-535.
- Cresswell, C.F. (1980). Increasing crop production through improved efficiency of photosynthesis and use of nitrogen fertilizer. *Sch. Afr. J. of Science*, 76: 107-116.
- Cresswell, C.F., Tew, A.J. and Lewis, O.A.M. (1977). The regulation of carbon metabolism in C_4 photosynthetic plants by inorganic nitrogen. In: Sixth Long Ashton Symposium Ed. Hewitt, E.J. and Cutting, C.V. pp. 4151-773. Academic Press, New York, London.
- Dailey, F.A., Kuo, T. and Warner, R.L. (1982). Pyridine nucleotide specificity of barley nitrate reductase. *Plant Physiol.*, 69: 1196-1199.
- Dale, J.E. (1979). Nitrogen supply and utilization in relation to development of the cereal seedling. In: Sixth Long Ashton Symposium Ed. Hewitt, E.J. and Cutting, C.V. pp. 153-163. Academic Press, New York, London.
- De Visser, R., Brouwer, K.S. and Posthumus, F. (1986). Alternative path mediated ATP synthesis in roots of *Pisum sativum* upon nitrogen supply. *Plant Physiol.*, 80: 295-300.
- Deane-Drummond, C.E. (1984a). Nitrate transport into *Chara corallina* cells using $^{36}\text{ClO}_3$ as an analogue for nitrate. *J. Experimental Bot.*, 35: 1289-1298.

Deane-Drummond, C.E. (1984b). Nitrate transport into Chara corallina cells using ClO_3^- as an analogue for nitrate. I Interaction between ClO_3^- and NO_3^- and characterisation of $\text{ClO}_3^-/\text{NO}_3^-$ influx. J. Experimental Bot., 35: 1290-1298.

Deane-Drummond, C.E. (1984c). Nitrate transport into Chara corallina cells using ClO_3^- as an analogue for nitrate. II Comparison with ^{14}C methylamine fluxes at different pH and $\text{NH}_4^+/\text{NO}_3^-$ interactions. J. Experimental Bot., 35: 1299-1308.

Deane-Drummond, C.E. (1984d). Mechanism of nitrate uptake into Chara corallina cells: lack of evidence for obligatory coupling to proton pump and a new $\text{NO}_3^-/\text{NO}_3^-$ exchange model. Plant, Cell and Environment: 7-317.

Deane-Drummond, C.E. (1983). Regulation of nitrate uptake into Chara corallina cells via NH_4^+ stimulation of NO_3^- efflux. Plant, Cell and Environment: 8-105.

Di Martino Rigano, V., Martello, A., Di Martino, C. and Rigano, C. (1985). Effect of CO_2 and phosphate deprivation on the control of nitrate, nitrite and ammonium metabolism in Chlorella. Physiol. Plant., 63: 241-246.

Doddams, H., Hofstra, J.J. and Feenstra, W.J. (1978). Uptake of nitrate by mutants of Arabidopsis thaliana, disturbed in uptake or reduction of nitrate. I Effect of nitrogen source during growth on uptake of nitrate and chlorate. Physiol. Plant., 43: 343-350.

Dry, I., Wallace, W. and Nicholas, D.J.D. (1981). Role of ATP in nitrite reduction in roots of wheat and pea. Planta, 152: 234-238.

Eaglesham, A.R.J. and Hewitt, E.J. (1971). The regulation of nitrate reductase activity from spinach (Spinacea oleracea L.) by thiol compounds in the presence of adenosine-5'-diphosphate. FEBS Lett 15-317.

Eisele, R. and Ullrich, W.R. (1977). Effect of g and CO_2 on nitrate uptake and coupled OH^- flux in Ankistrodesmus braunii. Plant. Physiol., 59: 18-21.

Elrifi, I.R. and Turpin, D.H. (1986). Nitrate and ammonium induced photosynthetic suppression in N-limited Selenastrum minutum. Plant Physiol., 81: 273-279.

Elrifi, I.R. and Turpin, D.H. (1987). The path of carbon flow during NO_3^- - induced photosynthetic suppression in N-limited Selenastrum minutum. Plant Physiol., 83: 97-104.

Engemann, J. and Brown, A.W. (1980). Regulation of dark CO_2 fixation in Avena coleoptila sections by α -naphthylacetate and ammonia. Plant Science Letters, 21: 269-274.

Espe, G.S. and Colman, B. (1986). Inorganic carbon uptake during photosynthesis I. A theoretical analysis using the isotopic disequilibrium technique. Plant Physiol., 80: 863-869.

Espe, G.S., Owttrim, G.W. and Colman, B. (1986). Inorganic carbon uptake during photosynthesis. II. Uptake by isolated Asparagus mesophyll cells during isotopic disequilibrium. Plant Physiol., 80: 870-876.

Fair, P., Tew, J. and Cresswell, C.F. (1974). Enzyme activities associated with carbon dioxide exchange in illuminated leaves of Hordeum vulgare L. III. Effects of concentration and form of nitrogen supplied on carbon dioxide compensation point. Ann. Bot., 38: 39-43.

Farquhar, G.D. and Sharkey, T.D. (1982). Stomatal conductance and photosynthesis. *Ann. Rev. Plant Physiol.*, 33: 317-345.

Felle, H. (1981). Stereospecificity and electrogenicity of amino acid transport in *Riccia fluitans*. *Planta*, 152: 505-512.

Ferrari, T.E., Yoder, O.C. and Filner, P. (1973). Anaerobic nitrate production by plant cells and tissues: Evidence for two nitrate pools. *Plant Physiol.*, 51: 423-431.

Frank, B. and Marek, M. (1983). The response of the net photosynthetic rate to irradiance in barley leaves as influenced by nitrogen supply. *Photosynthetica*, 17: 572-577.

Flügge, U.I. and Heldt, H.W. (1976). Identification of a protein involved in phosphate transport of chloroplasts. *FEBS Letters*, 68: 259-262.

Flügge, U.I. and Heldt, H.W. (1977). Specific labelling of a protein involved in phosphate transport of chloroplasts by pyridoxal-5'-phosphate. *FEBS Letters*, 82: 29-33.

Flügge, U.I. and Heldt, H.W. (1978). Specific labelling of the active site of the phosphate translocator in spinach chloroplasts by 2, 4, 6-trinitrobenzene sulfonate. *Biochemical and Biophysical Research Communications*, 84: 37-44.

Franz, S.L. and Tattar, T.A. (1981). Effect of sugar and amino acids on membrane potential in two clones of sugarcane. *Plant Physiol.*, 67: 150-155.

Fuggi, A., Di Martino Rigano, V., Vona, V. and Rigano, C. (1981). Nitrate and ammonium assimilation in algal cell-suspensions and related pH variations in the external medium monitored by electrodes. *Plant Science Letters*, 23: 129-138.

Fuggi, A. (1985). Mechanism of proton-linked nitrate uptake in *Cyanidium caldarium*, an acidophilic non-vacuolate alga. *Biochimica et Biophysica Acta*, 815: 392-398.

Garland, P.B., Downie, J.A. and Haddock, B.A. (1975). Proton translocation and the respiratory nitrate reductase of *Escherichia coli*. *Biochem. J.*, 152: 547-559.

Gimmler, H. (1977). Photophosphorylation *in vivo*. In: *Encyclopedia of Plant Physiology*, New Series, Vol. 5. Photosynthesis I. Ed. Trebst, A., and Avron, M. Springer Verlag, Heidelberg.

Glass, A.M., Thompson, R.G. and Bordeleau, L. (1985). Regulation of NO_3^- influx in Barley. Studies using $^{15}\text{NO}_3^-$. *Plant Physiol.*, 77: 379-381.

Goldberg, R., Thor, V., Dubacq, J-P., Prat, R. and Pierron, M. (1986). Time course of a differentiation process during isolation and culture of protoplasts: comparison between protoplasts from young and mature mung bean hypocotyl. *Plant Science*, 46: 63-68.

Goldsmith, J., Livoni, J.P., Norberg, C.L. and Segel, I.H. (1973). Regulation of nitrate uptake in *Penicillium chrysogenum* by ammonium ion. *Plant Physiol.*, 52: 362-367.

Golterman, H.L. (1969). *Methods for chemical analysis of fresh waters*. IBP Handbook No8. Blackwell Scientific Publications, Edinburgh.

Grant, B.R., Atkins, C.A. and Calvin, D.T. (1970). Intracellular location of nitrate reductase and nitrite reductase in spinach and sunflower leaves. *Planta*, 94: 60-72.

Gray, V.M. (1984). Nitrogen assimilation in chlorophyllous and non-chlorophyllous tissue. PhD thesis, University of the Witwatersrand, Johannesburg.

Gray, V.M. and Cresswell, C.F. (1983). Anaerobic stimulation of nitrate utilization in sterile excised Zea mays L. roots. *Stn. Afr. J. of Science*, 79: 73.

Gray, V.M. and Cresswell, C.F. (1983). Nitrate utilization and nitrite accumulation by Zea mays L. roots under anaerobic and aerobic conditions as influenced by nitrate levels. *Z. Pflanzenphysiol. Bd.*, 110: 439-446.

Gray, V.M. and Cresswell, C.F. (1984). The effect of inhibitors of photosynthetic and respiratory electron transport on nitrate reduction and nitrite accumulation in excised Z. mays leaves. *J. Experimental Bot.*, 35: 1166-1176.

Gray, V.M. and Cresswell, C.F. (1984). Nitrite utilization by excised Zea mays L. roots under anaerobic conditions. *Plant Science Letters*, 33: 31-38.

Griffith, C.J., Rea, P.A., Blumwald, E. and Poole, R.J. (1986). Mechanism of stimulation and inhibition of tonoplast H^+ -ATPase of Beta vulgaris by chloride and nitrate. *Plant Physiol.*, 81: 120-125.

Guerrero, M.G., Vega, J.M. and Losada, M. (1981). The assimilatory nitrate-reducing system and its regulation. *Ann. Rev. Plant Physiol.*, 32: 169-204.

Gupta, S.C. and Beevers, L. (1985). Regulation of synthesis of nitrite reductase in pea leaves: In vivo and in vitro studies. *Planta*, 166: 89-95.

Hall, N.P., Reggiani, R., Franklin, J., Keys, A.J. and Lea, P.J. (1984). An investigation into the interaction between nitrogen nutrition, photosynthesis and photorespiration. *Photosynthesis Research*, 5: 361-369.

Hall, A.E. and Schulze, E.D. (1980). Stomatal response to environment and a possible interaction between stomatal effects on transpiration and CO_2 assimilation. *Plant, Cell and Environment*, 3: 467-474.

Hampp, R. (1985). Triosephosphates modulate leaf mitochondrial phosphorylation by inhibition and uncoupling of electron transport. *Plant Physiol.*, 79: 690-694.

Haynes, R.J. and Goh, K.M. (1978). Ammonium and nitrate nutrition of plants. *Biol. Rev.*, 53: 465-510.

Heber, U. (1974). Metabolite exchange between chloroplasts and cytoplasm. *Ann. Rev. Plant Physiol.*, 25: 393-421.

Heber, U. and Heldt, H.W. (1981). The chloroplast envelope: structure, function and role in leaf metabolism. *Ann. Rev. Plant Physiol.*, 32: 139-168.

Heldt, H.W. (1976). Transport of metabolites between the cytoplasm and the mitochondrial matrix. In: *Encyclopedia of Plant Physiology*, New Series, Vol. 3. Ed. Stocking, C.R. and Heber, U., pp. 235-254, Springer Verlag, New York.

Heldt, H.W., Chon, C.J., Maronde, D., Harold, A., Stankovic, Z.S., Walker, D.A., Kraminer, A., Kirk, M.R. and Heber, U. (1977). Role of orthophosphate and other factors in the regulation of starch formation in leaves and isolated chloroplasts. *Plant Physiol.* 59: 1146-1155.

Heldt, H.W. and Sauer, F. (1971). The inner membrane of the chloroplast envelope as the site of specific metabolite transport. *Biochimica et Biophysica Acta*, 234: 83-91.

Heldt, H.W. and Rapley, L. (1970). Specific transport of inorganic phosphate, 3-phosphoglycerate and dihydroxyacetonephosphate and of dicarboxylates across the inner membrane of spinach chloroplasts. *FEBS Letters*, 10: 143-148.

Hewitt, E.J. (1952). Sand and water culture methods used in the study of plant nutrition. Commonwealth Bureau Horti. Plantac. Crops Tech. Commun., 22. East Malling, UK.

Hewitt, E.J. (1975). Assimilatory nitrate-nitrite reduction. *Ann. Rev. Plant Physiol.*, 26: 73-100.

Hewitt, E.J., Huckleby, D.P. and Notton, B.A. (1976). Nitrate metabolism. In: *Plant Biochemistry*. Ed Bonner, J., and Varner, J., pp. 663-681. Academic Press, New York, London.

Hewitt, E.J., Huckleby, D.P., Mann, A.F., Notton, B.A. and Rucklidge, G.J. (1979). Regulation of nitrate assimilation in plants. In: *Nitrogen assimilation of plants*, pp. 255-287. Ed. Hewitt, E.J. and Cutting, C.V. Academic Press, New York, London.

Hipkin, C.R. and Cannons, A.C. (1985). Inorganic nitrogen assimilation and the regulation of glucose 6-phosphate dehydrogenase in unicellular algae. *Plant Science*, 41: 155-160.

House, C.M. and Anderson, J.W. (1980). Light dependent reduction of nitrate by pea chloroplasts in the presence of nitrate reductase and C₄-dicarboxylic acids. *Phytochemistry*, 19: 1925-1930.

Huckleby, D.P., James, D.M., Bandwell, M.J. and Hewitt, E.J. (1976). Properties of nitrite reductase from Cucurbita pepo. *Phytochemistry*, 15: 599-603.

Huber, S.C., Kerr, P.S. and Ruffy, T.W. (1985). Diurnal changes in sucrose phosphate synthase activity in leaves. *Physiol. Plant.*, 64: 81-87.

Hunt, E.R., Weber, J.A. and Gates, D.M. (1985a). Effects of nitrate application on Amaranthus powellii Wats. I. Changes in photosynthesis, growth rates and leaf area. *Plant Physiol.*, 79: 614-618.

Hunt, E.R., Weber, J.A. and Gates, D.M. (1985b). Effects of nitrate application on Amaranthus powellii Wats. II. Stomatal response to vapor pressure difference is consistent with optimization of stomatal conductance. *Plant Physiol.*, 79: 614-618.

Hunt, E.R., Weber, J.A. and Gates, D.M. (1985c). Effects of nitrate application on Amaranthus powellii Wats. III. Optimal allocation of leaf nitrogen for photosynthesis at stomatal conductance. *Plant Physiol.*, 79: 614-618.

Ikeda, M., Ogren, W.L. and Hageman, R.H. (1984). Effect of methionine sulfoximine on photosynthetic carbon metabolism in wheat leaves. *Plant and Cell Physiol.*, 25: 447-452.

Ishiyama, Y., Shinoda, I., Fukushima, K. and Tamura, G. (1985). Some properties of ferredoxin-nitrite reductase from green shoots of bean and an immunological comparison with nitrite reductase from roots and etiolated shoots. *Plant Science*, 39: 89-95.

Ivanova, N.N. and Drobysheva, N.I. (1982). Superoxide radical in nitrate reduction by plant peroxidase. *Soviet Plant Physiol.*, 29: 385-389.

Ivanova, N.N. and Drobysheva, N.I. (1985). Participation of oxygen anion radical in enzymatic reactions of nitrate reduction. *Soviet Plant Physiol.*, 32: 386-391.

Izawa, S. (1977). Inhibitors of electron transport. In: *Encyclopedia of Plant Physiology*, New Series, Vol. 5. Photosynthesis I. Ed. Trebst, A., and Avron, M., pp. 266-282, Springer Verlag, Heidelberg.

Jackson, W.A., Volk, R.J., Morgan, M.A., Pan, W.L. and Tayker, R.H. (1986). Nitrogen uptake and partitioning by roots. In press.

Jolly, S.O. and Tolbert, N.E. (1978). NADH-Nitrate reductase inhibitor from spinach leaves. *Plant Physiol.*, 62: 197-203.

Joy, K.W. and Hageman, R.H. (1966). The purification and properties of nitrite reductase from higher plants, and its dependence on ferredoxin. *Biochem.*, 100: 263-273.

Kaiser, G. and Heber, U. (1983). Photosynthesis of leaf cell protoplasts and permeability of the plasmalemma to some solutes. *Planta*, 157: 462-470.

Kaiser, J.J. and Lewis, O.A.M. (1980). Nitrate-nitrogen assimilation in the leaves of *Helianthus annuus* L. *New Phytol.*, 85: 235-241.

Kaplan, D. and Lips, S.H. (1984). A comparative study of nitrate reduction and the oxidation of glycolate. *Israel J. Bot.*, 33: 1-11.

Kaplan, D., Meyer, A.M. and Lips, S.H. (1984). A constitutive component of nitrate reductase in barley leaves. *Israel J. Bot.*, 33: 13-23.

Kayshap, A.H. and Singh, D.P. (1985). Ammonium transport in unicellular cyanobacterium *Anacystis nidulans*. *J. Plant Physiol.*, 121: 319-330.

Kayupova, G.A., Klyshev, L.K. and Rakova, N.M. (1983). Role of superoxide dismutase in the formation of nitrite in pea roots in the presence of salinization of the medium. *Soviet Plant Physiol.*, 30: 116-120.

Kendall, A.C., Wallsgrove, R.M., Hall, N.P., Turner, J.C. and Lea, P.J. (1986). Carbon and nitrogen metabolism in barley (*Hordeum vulgare* L.) mutants lacking ferredoxin-dependent glutamate synthase. *Planta*, 168: 316-323.

Kerr, P.S., Huber, S.G. and Israel, D.W. (1984). Effect of N-source on soybean leaf sucrose phosphate synthase, starch formation, and whole plant growth. *Plant Physiol.*, 75: 483-488.

Keys, A.J., Bird, I.E. and Cornelius, M.J. (1978). Photorespiratory nitrogen cycle. *Nature*, 275: 741-742.

Kinraide, T.B. and Etherton, B. (1980). Electrical evidence for different mechanisms of uptake for basic, neutral, and acidic amino acids in oat coleoptiles. *Plant Physiol.*, 65: 1085-1089.

- Kinraide, T.B., Newman, I.A. and Etherton, B. (1984). A quantitative simulation model for H^+ -amino acid cotransport to interpret the effects of amino acids on membrane potential and extracellular pH. *Plant Physiol.*, 76: 806-813.
- Kirkby, E.A. and Armstrong, M.J. (1980). Nitrate uptake by roots as regulated by nitrate assimilation in the shoot of castor oil plants. *Plant Physiol.*, 65: 286-290.
- Kirkby, E.A. and Knight, A.R. (1977). Influence of the level of nitrate nutrition on ion uptake and assimilation, organic acid accumulation, and cation-anion balance in whole tomato plants. *Plant Physiol.*, 60: 349-353.
- Klaus, R.E., Berger, M.G. and Fock, H.P. (1985). Effect of light intensity on ammonia assimilation in maize leaves. *Photosynthesis Research*, 6: 221-228.
- Klepper, L., Flesher, D. and Hageman, R.H. (1971). Generation of reduced nicotinamide adenine dinucleotide for nitrate reduction in green leaves. *Plant Physiol.*, 48: 580-590.
- Kow, Y., Erbes, D.L. and Gibbs, M. (1982). Chloroplast respiration. A means of supplying oxidized pyridine nucleotide for dark chloroplastic metabolism. *Plant Physiol.*, 69: 442-447.
- Lamaze, T., Sentenac, H. and Grignon, C. (1984). Effects of nitrate on phosphate accumulation and transport by corn roots. *Physiol. Veg.*, 22: 155-161.
- Larsson, M., Larsson, C.-M. and Guerrero, M.G. (1985a). Photosynthetic nitrogen metabolism in high and low CO_2 -adapted *Scenedesmus*. *J. Experimental Bot.*, 36: 1373-1386.
- Larsson, M., Olsson, T. and Larsson, C.-M. (1985b). Distribution of reducing power between photosynthetic carbon and nitrogen assimilation in *Scenedesmus*. *Planta*, 164: 246-253.
- Lea, P.J. and Miflin, B.J. (1979). Photosynthetic ammonia assimilation. In: *Encyclopedia of Plant Physiology, Photosynthesis II*, New Series, Vol. 6, Gibbs, M. and Latzko, E., pp. 445-456, Springer Verlag, Berlin.
- Lea, R.B. (1978). Inorganic nitrogen metabolism in barley roots under poorly aerated conditions. *J. Experimental Bot.*, 29: 693-708.
- Leegood, R.C., Edwards, G.E. and Walker, D.A. (1982). Chloroplasts and protoplasts. In: *Techniques in bioproductivity and photosynthesis*. Ed. Coombs, J. and Hall, D.O., pp. 94-111, Pergamon Press, Oxford.
- Lehninger, A.L. (1976). *Biochemistry*. Worth, New York.
- Lew, R.R. and Spanswick, R.M. (1984). Proton-pumping activities of soybean (*Glycine max* L.) root microsomes: localization and sensitivity to nitrate and vanadate. *Plant Science Letters*, 36: 187-193.
- Lew, R.R. and Spanswick, R.M. (1985). Characterization of anion effects on the nitrate-sensitive ATP-dependent proton pumping activity of soybean (*Glycine max* L.) seedling root microsomes. *Plant Physiol.*, 77: 352-357.

Lewis, O.A.M. and Chadwick, S. (1983). An ^{15}N investigation into nitrogen assimilation in hydroponically-grown barley (*Hordeum vulgare* L. cv. Clipper) in response to nitrate, ammonium and mixed nitrate and ammonium nutrition. *New Phytol.*, 55: 635-646.

Lewis, O.A.M., Chadwick, S. and Withers, J. (1983). The assimilation of ammonium by barley roots. *Planta*, 159: 483-486.

Lips, S.H. (1971). Photorespiration and nitrate reduction. In: IInd International Congress on Photosynthesis. Strassburg, pp. 2241-2249.

Lips, S.H. (1977). Photosynthesis and Photorespiration in Nitrate Metabolism. In: Sixth Long Ashton Symposium. Ed. Hewitt, E.J. and Cutting, C.V. Academic Press, New York, London.

Lips, S.H. and Avissar, Y. (1972). Plant leaf microbodies as the intracellular site of nitrate reductase and nitrite reductase. *Eur. J. Biochem.*, 29: 20-24.

Loyola-Vargas, V.M. and De Jimenez, E.S. (1984). Differential role of glutamate dehydrogenase in nitrogen metabolism in maize tissues. *Plant Physiol.*, 76: 536-540.

MacKown, C.T., Jackson, W.A. and Volk, R.J. (1982). Restricted nitrate influx and reduction in corn seedlings exposed to ammonium. *Plant Physiol.*, 69: 353-359.

MacKown, C.T., Jackson, W.A. and Volk, R.J. (1983). Partitioning of previously accumulated nitrate to translocation, reduction, and efflux in corn roots. *Planta*, 157: 8-14.

Megalhaes, A.C., Neyra, C.A. and Hageman, R.H. (1974). Nitrite Assimilation and Amino Nitrogen Synthesis in Isolated Spinach Chloroplasts. *Plant Physiol.*, 53: 411-415.

Mann, A.F., Hucklesby, D.P. and Hewitt, E.J. (1978). Sources of reducing power for nitrate reduction in spinach leaves. *Planta*, 140: 261-263.

Mann, A.F., Hucklesby, D.P. and Hewitt, E.J. (1979). Effect of aerobic and anaerobic conditions on the *in vivo* nitrate reductase assay in spinach leaves. *Planta*, 146: 83-86.

Marcus, Y., Volokita, M. and Kaplan, A. (1984). The location of the transporting system for inorganic carbon and the nature of the form translocated in *Chlamydomonas reinhardtii*. *J. Experimental Bot.*, 35: 1136-1144.

Marek, M. (1984). The effect of nitrogen nutrition and photon fluence rate on the oxygen dependence of CO_2 compensation concentration and mitochondrial respiration in the light in young barley leaves. *Photosynthetica*, 18: 43-49.

Marek, M. and Frank, R. (1984). Effect of nitrogen supply on net photosynthetic rate in barley leaves. *Photosynthetica*, 18: 219-225.

Martin, E.S., Donkin, M.E. and Stevens, R.A. (1983a). Stomata. Edward Arnold, Southampton.

Martin, F., Winspear, M.J., MacFarlane, J.D. and Oaks, A. (1983b). Effect of methionine sulfoximine on the accumulation of ammonia in C_3 and C_4 leaves. *Plant Physiol.*, 71: 177-181.

Martinoia, E., Heck, U. and Wiemken, A. (1981). Vacuoles as storage compartments for nitrate in barley leaves. *Nature*, 289: 292-293.

Mengel, K. and Schubert, S. (1985). Active extrusion of protons into deionized water by roots of intact maize plants. *Plant Physiol.*, 79: 344-348.

Mende, D. and Wessner, W. (1985). Bicarbonate *in vivo* requirement of photosystem II in the green alga *Chlorella obotrys stellata*. *J. Plant Physiol.*, 118: 259-266.

Mengel, K., Robin, P. and Meisner, L. (1983). Nitrate reductase activity in shoots and roots of maize seedlings as affected by the form of nitrogen nutrition and the pH of the nutrient solution. *Plant Physiol.*, 71: 618-622.

Miflin, B.J. (1974). Nitrite reduction in leaves; studies on isolated chloroplasts. *Planta*, 116: 187-196.

Morgan, M.A., Jackson, W.A. and Volk, R.J. (1985a). Concentration dependence of the nitrate assimilation pathway in maize roots. *Plant Science*, 38: 185-191.

Morgan, M.A., Jackson, W.A. and Volk, R.J. (1985b). Uptake and assimilation of nitrate by corn roots during and after induction of the nitrate uptake system. *J. Experimental Bot.*, 36: 859-869.

Morot-Gaudry, J-F., Thuillier, F., Lesaint, C., Chaillou, S. and Jolivet, E. (1985). Is ^{14}C photodistribution in C_4 acids of maize leaves affected by type of nitrogen supply? *Physiol. Vég.*, 23: 6-11.

Mouriaux, G. and Douce, R. (1981). Slow passive diffusion of orthophosphate between intact isolated chloroplasts and suspending medium. *Plant Physiol.*, 67: 470-473.

Naik, M.S. and Nicholas, D.J.D. (1981). Relation between CO_2 evolution and *in situ* reduction of nitrate in wheat leaves. *Aust. J. Plant Physiol.*, 8: 515-524.

Naik, M.S., Abrol, Y.P., Nair, T.V.R. and Ramarao, C.S. (1982). Nitrate assimilation - its regulation and relationship to reduced nitrogen in higher plants. *Phytochemistry*, 21: 495-504.

Nelson, M. and Ilan, I. (1969). Inhibition of nitrate reductase from tomato leaves by adenosine-5'-diphosphate. *Plant and Cell Physiol.*, 10: 143-148.

Neyra, C. A. and Hageman, R. H. (1974). Dependence of nitrate reduction on electron transport in chloroplasts. *Plant Physiol.*, 54: 480-483.

Neyra, C.A. and Hageman, R.H. (1978). Pathway for nitrate assimilation in corn (*Zea mays* L.) leaves. *Plant Physiol.*, 62: 618-621.

Novacky, A. and Fischer, E. (1978). Membrane potential changes during transport of glycine as a neutral amino acid and nitrate in *Lemna gibba* G1. *FEBS Letters*, 88: 264-267.

O'Neill, S.D., Bennett, A.B. and Spanswick, R.M. (1983). Characterization of a NO_3^- -sensitive H^+ -ATPase from corn roots. *Plant Physiol.*, 72: 837-846.

Oaks, A. (1979). Nitrate reductase in roots and its regulation. In: Nitrogen assimilation of plants. Sixth Long Ashton Symposium, Ed. Hewitt, E.J., and Cutting, C.V., pp. 227-244, Academic Press, New York, London.

Oji, Y., Watanabe, M., Wakiuchi, N. and Okamoto, S. (1985). Nitrite reduction in barley-root plastids: dependence on NADPH coupled with glucose-6-phosphate and 6-phosphogluconate dehydrogenases, and possible involvement of an electron carrier and a diaphorase. *Planta*, 165: 85-90.

Osman, M.Z.H., Metzner, H. and Fischer, K. (1982). Effects of nitrate on thylakoid reactions I. Influence on photosynthetic electron transport. *Photosynthetica*, 16: 7-12.

Pan, W.L., Jackson, W.A. and Moll, R.H. (1985). Nitrate uptake and partitioning by corn root systems. *Plant Physiol.*, 77: 560-566.

Platt, S.G., Plaut, Z. and Bassham, J.A. (1977). Ammonia regulation of carbon metabolism in photosynthesizing leaf discs. *Plant Physiol.*, 167: 739-472.

Plaut, Z., Lendzian, K. and Bassham, J.A. (1977). Nitrite reduction in reconstituted and whole spinach chloroplasts during carbon dioxide reduction. *Plant Physiol.*, 59: 184-188.

Puranik, R.M. and Srivastava, H.S. (1983). Increase in nitrate reductase activity in the presence of sucrose in bean leaf segments. *Phytochemistry*, 22: 2383-2387.

Purczeld, P., Chon, C.J., Portis, A.R., Heldt, H.W. and Heber, U. (1978). The mechanism of the control of carbon fixation by the pH in the chloroplast stroma: studies with nitrate-mediated proton transfer across the envelope. *Biochimica et Biophysica Acta*, 501: 488-494.

Radin, J.W. (1973). In vivo assay of nitrate reductase in cotton leaf disks. *Plant Physiol.*, 51: 332-336.

Radin, J.W., Parker, L.L. and Sell, C.R. (1978). Partitioning of sugar between growth and nitrate reduction in cotton roots. *Plant Physiol.*, 62: 550-553.

Radmer, R.J. and Kok, B. (1976). Photoreduction of O_2 primes and replaces CO_2 assimilation. *Plant Physiol.*, 59: 184-188.

Ramamo, C.S., Srinivasan, and Naik, M.S. (1981). Origin of reductant for reduction of nitrate and nitrite in rice and wheat leaves in vivo. *New Phytol.*, 87: 517-525.

Rathnam, C.K.M. (1978). Malate and dihydroxyacetone phosphate-dependent nitrate reduction in spinach leaf protoplasts. *Plant Physiol.*, 62: 220-223.

Rausch, T., Ziemann-Roth, M. and Hilgenberg, W. (1985). ADP is a competitive inhibitor of ATP-dependent H^+ transport in microsomal membranes from Zea mays L. coleoptiles. *Plant Physiol.*, 77: 881-885.

Raven, J.A. and Smith, F.A. (1976). Nitrogen assimilation and transport in vascular land plants in relation to intracellular pH regulation. *New Phytol.*, 76: 415-431.

Redinbaugh, M. and Campbell, W.H. (1981). Purification and characterization of NAD(P)H:nitrate reductase and NADH:nitrate reductase from corn roots. *Plant Physiol.*, 68: 115-120.

Read, A.J., Calvin, D.T., Sherrard, J.H. and Hageman, R.H. (1983). Assimilation of $[^{15}N]$ nitrate and $[^{15}N]$ nitrite in leaves of five plant species under light and dark conditions. *Plant Physiol.*, 71: 291-294.

Reggiani, R., Brambilla, I. and Bertani, A. (1985). Effect of exogenous nitrate on anaerobic metabolism in excised rice roots. J. Experimental Bot., 36: 1698-1704.

Reinhold, L., Volokita, M., Zenvirth, D. and Kaplan, A. (1984). Is HCO_3^- transport in Anabaena a Na symport? Plant Physiol., 76: 1090-1092.

Reisenauer, H.M. (1978). Absorption and utilization of ammonium nitrogen by plants. In: Nitrogen in the Environment. Ed. Nielson, D.R. and MacDonald, J.G. Academic Press, New York, London.

Rigano DiMartino, V., Martello, A., DiMartino, C. and Rigano, C. (1985). Effect of CO_2 and phosphate deprivation on the control of nitrate, nitrite and ammonium metabolism in Chlorella. Physiol. Plant., 63: 241-246.

Robin, P., Streit, L., Campbell, W.H. and Harper, J.E. (1985). Immunochemical characterization of nitrate reductase forms from wild-type (cv Williams) and NR mutant soybean. Plant Physiol., 77: 232-236.

Robinson, J.M. (1986). Carbon dioxide and nitrite photoassimilatory processes do not intercompete for reducing equivalents in spinach and soybean leaf chloroplasts. Plant Physiol., 80: 676-684.

Robinson, S.P. and Beevers, H. (1981). Evidence for amino acid:proton cotransport in Ricinus cotyledons. Planta, 152: 527-533.

Robinson, S.P. and Lovays, B.R. (1986). Uptake and retention of external solutes from the digest medium during preparation of protoplasts. Plant Science, 46: 43-51.

Romani, G., Marre, T.M., Bellando, M., Alloatti, G. and Marre, E. (1985). H^+ Extrusion and potassium uptake associated with potential hyperpolarization in maize and wheat root segments treated with weak permeant acids. Plant Physiol., 79: 734-739.

Roth-Bejerano, N. and Lips, S.H. (1973). Glycolate dehydrogenase activity in higher plants. Israel J. Bot., 22: 1-7.

Ruffy, T.W., Israel, D.W. and Volk, R.J. (1984). Assimilation of $^{15}\text{NO}_3^-$ taken up by plants in the light and in the dark. Plant Physiol., 76: 769-775.

Sahulka, J. (1981). The effect of some carbohydrates on nitrite reductase level in isolated pea roots. Plant Science Letters, 23: 169-75.

Salvucci, M.E. (1983). Mechanisms for reducing photo aspiration in submersed aquatic angiosperms. PhD Thesis, University of Florida.

Sauer, N. (1984). A general amino acid permease is inducible in Chlorella vulgaris. Planta, 161: 425-431.

Sauer, N., Komor, E. and Tanner, W. (1983). Regulation and characterization of two inducible amino acid transport systems in Chlorella vulgaris. Planta, 159: 404-410.

Sawada, S., Misawa, T. and Miyachi, S. (1984). Effects of nutritional levels of phosphate on photosynthetic rate and Hill reaction activity determined successively with the same leaf slices obtained from single rooted leaf of dwarf bean. Photosynthetica, 18: 541-548.

Sawhney, S.K., Naik, M.S. and Nicholas, D.J.D. (1978a). Regulation of NADH supply for nitrate reduction in green plants via photosynthesis and mitochondrial respiration. Biochemical and Biophysical Research Communications, 81: 1209-1216.

Sawhney, S.K., Naik, M.S. and Nicholas, D.J.D. (1978b). Regulation of nitrate reduction by light, ATP and mitochondrial respiration in wheat leaves. Nature, 272: 647-648.

Schlee, J., Cho, B-H. and Komor, E. (1985). Regulation of nitrate uptake by glucose in *Chlorella*. Plant Science, 39: 25-30.

Scheiner, D. (1975). Determination of ammonia in solution. Water Res., 10: 31-36.

Schweizer, P. and Brismann, K.H. (1985). Effect of nitrate and ammonium nutrition of non-nodulated *Phaseolus vulgaris* L. on phosphoenolpyruvate carboxylase and pyruvate kinase activity. Plant Physiol., 78: 455-458.

Selga, M. (1983). Photosynthate export from chloroplasts under various irradiance and mineral nutrition. Photosynthetica, 17: 168-170.

Sentenac, H. and Grignon, C. (1985). Effect of pH on orthophosphate uptake by corn roots. Plant Physiol., 77: 136-141.

Shieh, Y.J. and Liao, W-Y. (1985). Effect of nitrogen on growth and photosynthesis of rice plants (*Oryza sativa* L.). Bot. Bull. Academia Sinica, 26: 127-146.

Shin, I.V., Chandylowa, L.V., Giller, Y.E. and Masyrov, Y.S. (1985). Effect of bicarbonate on the Hill reaction and photophosphorylation in pea and cotton chloroplasts. Soviet Plant Physiol., 32: 633-637.

Skokut, T.A., Wolk, C.P., Thomas, J., Meeks, J.C., Shaffer, P.W. and Chen, W.-S. (1978). Initial organic products of assimilation of [15 N]Ammonium and [15 N]Nitrate by tobacco cells cultured on different sources of nitrogen. Plant Physiol., 62: 299-304.

Smirnov, N. and Stewart, G.R. (1985). Nitrate assimilation and translocation by higher plants: comparative physiology and ecological consequences. Physiol. Plant., 64: 133-140.

Smith, F.A. and Raven, J.A. (1979). Intracellular pH and its regulation. Ann. Rev. Plant Physiol., 30: 289-311.

Soares, M.I.M., Lips, S.H. and Crasswell, C.F. (1985a). Regulation of nitrate and nitrite reduction in barley leaves. Physiol. Plant., 64: 492-500.

Soares, M.I.M., Lips, S.H. and Crasswell, C.F. (1985b). Endogenous nitrate loss as an assay for nitrate reduction in vivo. Physiol. Plant., 64: 487-491.

Stenler, A. and Murphy, J.B. (1985). Bicarbonate-reversible and irreversible inhibition of photosystem II by monovalent anions. Plant Physiol., 77: 974-977.

Stepanova, A.M., Shumilova, A.A. and Fedoseenko, A.A. (1980). Influence of oxygen and nitrate on assimilation of $^{14}\text{CO}_2$ by corn leaves. Soviet Plant Physiol., 27: 12-16.

Stitt, M. and Heldt, H.W. (1985). Control of photosynthetic sucrose synthesis by fructose 2, 6-bisphosphate. *Plant Physiol.*, 79: 599-608.

Stitt, M., Lilley, R. and Heldt, H.W. (1982). Adenine nucleotide levels in the cytosol, chloroplasts, and mitochondria of wheat leaf protoplasts. *Plant Physiol.*, 70: 971-977.

Stitt, M., Wirtz, W. and Heldt, H.W. (1983). Regulation of sucrose synthesis by cytoplasmic fructose biphosphatase and sucrose phosphate synthase during photosynthesis in varying light and carbon dioxide. *Plant Physiol.*, 72: 767-774.

Sugiyama, T., Mizuno, M. and Hayashi, M. (1984). Partitioning of nitrogen among Ribulose-1, 5-bisphosphate carboxylase/oxygenase, phosphoenolpyruvate carboxylase, and pyruvate dikinase as related to biomass productivity in maize seedlings. *Plant Physiol.*, 75: 665-669.

Suzuki, A., Oaks, A., Jacquet, J., Vidal, J. and Gadal, P. (1985). An electron transport system in maize roots for reactions of glutamate synthase and nitrite reductase. *Plant Physiol.*, 78: 374-378.

Ta, T.C. and Joy, K.W. (1984). Transamination, deamination, and the utilisation of asparagine amino nitrogen in pea leaves. *Can. J. Bot.*, 63: 881-884.

Tew, A.J. (1976). A study of the influence of nitrogen on the primary products and enzymes systems associated with carbon dioxide exchange in illuminated leaves of C₃ and C₄ photosynthetic plants. PhD Thesis, University of the Witwatersrand; Johannesburg.

Tew, J., Grossman, E.S., Fair, P. and Cresswell, C.F. (1974). A study of the fine structure, enzyme activities and pattern of ¹⁴CO₂ incorporation of highveld grasses from different successional stages. *Proc. Grassld. Soc. Sth. Afr.*, 9: 95-104.

Tobin, A. K. and Givan, C.V. (1984). Adenine nucleotide regulation of malate oxidation in isolated mung bean hypocotyl mitochondria. *Plant Physiol.*, 76: 21-25.

Tomati, U., Giovannozzi-Sermanni, G., Durpf, S. and Canella, C. (1976). MADE:Nitrate reductase activity restoration by rhodanese. *Phytochemistry*, 15: 597-598.

Ullrich, W.R. and Kunz, G. (1984). Effect of abscisic acid on nitrate, respiration and photosynthesis in green algae. *Plant Science Letters*, 37: 9-14.

Ullrich, W.R. and Novacky, A. (1981). Nitrate-dependent membrane potential changes and their induction in Lemna gibba G l. *Plant Science Letters*, 22: 211-217.

Underhill, P.A. (1977). Nitrate uptake kinetics and clonal variability in the neritic diatom Biddulphia aurita. *J. Phycol.*, 13: 170-176.

Vaklinova, S., Fedina, I., Vassilava, V. and Ananieva, L. (1981). Effect of nitrate and ammonium nitrogen on the intensity of photosynthesis's photorespiration and on the ribulose-1, 5-diphosphate carboxylase/oxygenase in C₃ and C₄ types of plants. *Photosynthesis VI*. pp. 55-62.

Van Beusichem, M.L., Bass, R. and Kirkby, E.A.N. (1985). Intercellular pH regulation during NO₂⁻ assimilation in shoot and roots of Ricinus communis. *Plant Physiol.*, 78: 768-773.

Van Rensen, J.J.S. and Snel, J.F.H. (1985). Regulation of photosynthetic electron transport by bicarbonate, formate and herbicides in isolated broken and intact chloroplasts. *Photosynthesis Research*, 6: 231-246.

Veen, B.W. and Kleinendorst, A. (1985). Nitrate accumulation and osmotic regulation in Italian ryegrass (*Lolium multiflorum* Lam.). *J. Experimental Bot.*, 36: 211-218.

Venkataramana, S. and Das, V.S.R. (1983). Interaction of Mehler-type reagents and electron transport inhibitors with chloroplast-localized, photosystem I based nitrite reductase. *Indian J. of Biochemistry and Biophysics*, 20: 170-172.

Walker, D.A. (1976). Plastids and Intracellular transport. In: *Encyclopedia of Plant Physiology*, New Series, Vol. 3. Transport in Plants. Ed. Stocking, C.R. and Heber, U., pp. 85-136, Springer Verlag, Heidelberg.

Wallsgrave, R.M., Keys, A.J., Lea, P.J. and Miflin, B.J. (1983). Photosynthesis, photorespiration and nitrogen metabolism. *Plant, Cell and Environment*: 6: 301-309.

Wallsgrave, R.M., Kendall, A.C., Hall, N.P., Turner, J.C. and Lea, P.J. (1986). Carbon and nitrogen metabolism in a barley (*Hordeum vulgare* L.) mutant with impaired chloroplast dicarboxylate transport. *Planta*, 168: 324-329.

Wintermans, J.F.G.M. and De Mots, A. (1965). Spectrophotometric characteristics of chlorophylls a and b and their pheophytins in ethanol. *Biochimica et Biophysica Acta*, 109: 448-453.

Woo, K.C. and Calvin, D.T. (1980). The role of malate in nitrate reduction in spinach leaves. *Can. J. Bot.*, 58: 517-521.

Wyse, R.E. and Komor, E. (1984). Mechanism of amino acid uptake by sugarcane suspension cells. *Plant Physiol.*, 76: 865-870.

Yoneyama, T. (1981). ^{15}N studies on the *in vivo* assay of nitrate reductase in leaves: occurrence of under-estimation of the activity due to dark assimilation of nitrate and nitrite. *Plant and Cell Physiol.*, 22: 1507-1520.

Yoneyama, T. (1984). ^{15}N Study of the dark assimilatory reduction of nitrate and nitrite in the leaf sections of chlorophyll mutants of rice (*Oryza sativa*). *Plant Science Letters*, 33: 195-200.

Yoneyama, T. and Kumazawa, K. (1975). A Kinetic study of the assimilation of ^{15}N -labelled nitrate in rice seedlings. *Plant and Cell Physiol.*, 16: 21-26.

Zocchi, G. (1985). Phosphorylation-dephosphorylation of membrane proteins controls the microsomal H^+ -ATPase activity of corn roots. *Plant Science*, 40: 153-159.

A: APPENDIX

Listing of program for calculation of photosynthetic rates written in Microsoft BASICA.

```
10 REM *** PHOTOSYNTHESIS ***
20 Q=1
30 CLS
40 DIM X(100), Y(100), PN(100)
50 LOCATE 12, 30
60 PRINT "PHOTOSYNTHESIS DATA MANAGER"
70 FOR T=1 TO 1000:NEXT T
80 CLS
90 INPUT "Do you wish to read an old file (Y/N)";Y$
100 IF Y$<>"Y" AND Y$<>"N" THEN PRINT :GOTO 90
110 IF Y$="N" GOTO 420
120 PRINT :PRINT :INPUT "Read from drive A or B (A/B)";Y$
130 IF Y$<>"A" AND Y$<>"B" THEN PRINT :GOTO 120
140 LET T$=Y$+";"
150 FILES T$
160 PR.#T :PRINT :INPUT "Which file to read ";Y$
170 IF RIGHT$(Y$, 3)="CHL" THEN LET L$="C"
180 OPEN Y$ FOR INPUT AS #1
190 GOTO 270
200 RESUME 210
210 CLS: LOCATE 12, 32
220 ON ERROR GOTO 0
230 PRINT "***** WARNING *****"
240 LOCATE 14, 32:PRINT "***** FILE ERROR *****"
250 FOR T=1 TO 500:NEXT T
260 CLS:GOTO 150
270 INPUT #1, Q
280 INPUT #1, START
290 INPUT #1, INC
300 FOR T=1 TO Q-1
310 INPUT #1, Y(T)
320 NEXT T
330 INPUT #1, P
340 INPUT #1, TP
350 INPUT #1, F
360 INPUT #1, NS
370 IF L$="C" GOTO 390
380 INPUT #1, A
390 CLOSE #1
400 IF L$="C" GOTO 710
410 GOTO 780
420 REM *** DATA ENTRY ***
430 CLS
440 LOCATE 12, 1
450 PRINT "ENTER THE DATA REQUESTED WHEN PROMPTED"
460 PRINT "-----"
470 PRINT :PRINT :INPUT "What are the time intervals between the readings ";INC
480 PRINT :PRINT :INPUT "Starting at time =";START
```

```

490 LET START=START-INC
500 CLS
510 PRINT :PRINT :INPUT "Enter the temperature in centigrade ";TP
520 LET TP=TP+273
530 PRINT :PRINT :INPUT "Enter the flow rate in litres per minute ";F
540 PRINT :PRINT :INPUT "Enter the atmospheric pressure in millibars ";P
550 PRINT :PRINT :INPUT "Enter the number of leaf area measurements you made
";NLA
560 CLS
570 FOR T=1 TO NLA
580 PRINT "Area ";T;" = ----- (cm2)"
590 INPUT LA
600 LET A=A+LA
610 NEXT T
620 LET A=A/(T-1)
630 PRINT :PRINT :INPUT "If you wish to type an explanatory note, do so now.
Otherwise type 'N' ";N$
640 IF N$="N" THEN LET N$=""
650 CLS
660 PRINT "ENTER THE NUMBER OF PPM"
670 PRINT "-----"
680 IF Y(Q)=9999 THEN END
690 PRINT :PRINT "Pn = ";PN(Q-1);" mg/dm2/hr for data point ";Q-1
700 PRINT :PRINT
710 PRINT "Enter data point ";Q
720 INPUT Y(Q)
730 IF Y(Q)=9999 AND Q>1 GOTO 780
740 IF Y(Q)=9999 THEN END
750 PN(Q)=(Y(Q)*.1)/(A/100)*((((F*1000)/60)*44)/22414)*(273/TP)*(P/1013)*361
760 LET Q=Q+1
770 GOTO 650
780 CLS
790 INPUT "Hard copy (Y/N)";H$
800 IF H$="Y" GOTO 1000
810 REM *** PRINT ***
820 PRINT :PRINT "READING #" TAB(14) "TIME" TAB(22) "ppm-CO2" TAB(33) "Value-Pn"
TAB(45) "INCREASE X"
830 PRINT "-----"
840 PRINT
850 FOR T=1 TO Q-1
860 PN(T)=(Y(T)*.1)/(A/100)*((((F*1000)/60)*44)/22414)*(273/TP)*(P/1013)*361
870 IF T=1 THEN D=CINT((PN(T)-PN(1))/PN(1))*100
880 PRINT T TAB(14) (T*INC)+START TAB(22) Y(T) TAB(33) CINT(PN(T)) TAB(45) D
890 NEXT T
900 PRINT :PRINT :PRINT :PRINT "Pn in mg/dm2/hr"
910 PRINT :PRINT "Pressure = ";P;" mB"
920 PRINT :PRINT "Temperature = ";TP;" K"
930 PRINT :PRINT "Flow rate = ";F;" l/min"
940 PRINT :PRINT "Leaf area = ";A;" cm2"
950 PRINT :PRINT :PRINT "Explanatory note : ";N$
960 PRINT :PRINT "Continue?"
970 Y$=INKEY$
980 IF Y$="" GOTO 970
990 GOTO 1130
1000 LPRINT :LPRINT "READING #" TAB(14) "TIME" TAB(22) "ppm-CO2" TAB(33) "Value-
Pn" TAB(45) "INCREASE X"
1010 LPRINT "-----"
1020 FOR T=1 TO Q-1

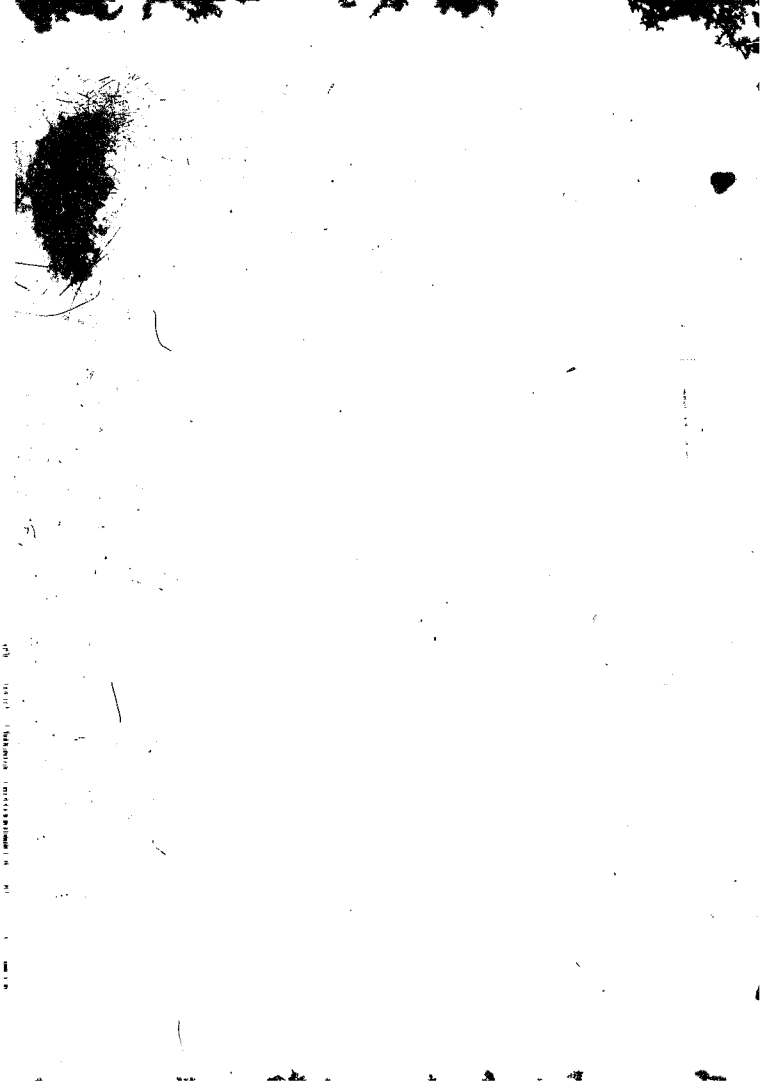
```

```

1030 PN(T)=(Y(T)*.1)/(A/100))*(((F*1000)/60)*.44)/22414)*(273/TP)*(P/1013)*.36!
1040 IF T=1 THEN D=0:ELSE D=CINT(((PN(T)-PN(1))/PN(1))*100)
1050 LPRINT T TAB(14) (T*INC)+START TAB(22) Y(T) TAB(33) CINT(PN(T)) TAB(45) D
1060 NEXT T
1070 LPRINT :LPRINT :LPRINT :LPRINT "Pn in mg/dm2/h"
1080 LPRINT :LPRINT "Pressure = ";P;" mB"
1090 LPRINT :LPRINT "Temperature = ";TP;" K"
1100 LPRINT :LPRINT "Flow rate = ";F;" l/min"
1110 LPRINT :LPRINT "Leaf area = ";A;" cm2"
1120 LPRINT :LPRINT :LPRINT "Explanatory note : "+N$
1130 REM ***DATA FILE***
1140 CLS
1150 INPUT "Do you wish to file your input data (Y/N) ";Y$
1160 IF Y$<>"Y" AND Y$<>"N" THEN PRINT :GOTO 1150
1170 IF Y$="N" GOTO 1410
1180 PRINT :PRINT :INPUT "Name for data file - Note .PIN will automatically be
appended ";M$
1190 IF LEN(M$)>8 GOTO 1130
1200 LET M$=M$+".PIN"
1210 ON ERROR GOTO 1260
1220 FILES M$
1230 CLS:LOCATE 12, 30:PRINT "*** WARNING - FILE EXISTS ***"
1240 LOCATE 15, 30:PRINT "ENTER A NEW FILE NAME"
1250 GOTO 1180
1260 RESUME 1270
1270 ON ERROR GOTO 0
1280 OPEN M$ FOR OUTPUT AS #1
1290 PRINT #1, Q
1300 PRINT #1, START
1310 PRINT #1, INC
1320 FOR T=1 TO Q-1
1330 PRINT #1, Y(T)
1340 NEXT T
1350 PRINT #1, P
1360 PRINT #1, TP
1370 PRINT #1, F
1380 PRINT #1, N$
1390 PRINT #1, A
1400 CLOSE #1
1410 PRINT :INPUT "Another look at data (Y/N)";Y$
1420 IF Y$<>"Y" AND Y$<>"N" GOTO 1410
1430 IF Y$="Y" GOTO 780
1440 REM *** OUTPUT FILE ***
1450 CLS
1460 INPUT "Do you wish to file your output values for further processing (Y/N)
";Y$
1470 IF Y$<>"Y" AND Y$<>"N" THEN PRINT :GOTO 1460
1480 IF Y$="N" GOTO 1530
1490 PRINT :PRINT :INPUT "Re-run program (Y/N) ";Y$
1500 IF Y$<>"Y" AND Y$<>"N" THEN PRINT :GOTO 1460
1510 IF Y$="N" THEN END
1520 RUN
1530 PRINT :PRINT :PRINT "Your data will be filed in two columns, Time and
Photosynthetic rates."
1540 PRINT :PRINT :INPUT "Enter a file name - note that .PRN will automatically
be appended. ";Y$
1550 IF LEN(M$)>8 GOTO 1540
1560 LET Y$=Y$+".PRN"
1570 ON ERROR GOTO 1620

```

1580 FILES Y\$
1590 CLS:LOCATE 12, 30:PRINT "*** WARNING - FILE EXISTS ***"
1600 LOCATE 15, 30:PRINT "ENTER A NEW FILE NAME"
1610 GOTO 1540
1620 RESUME 1630
1630 ON ERROR GOTO 0
1640 OPEN Y\$ FOR OUTPUT AS #1
1650 FOR T=1 TO Q-1
1660 PRINT #1, START+(INC*T), FN(T)
1670 NEXT T
1680 CLOSE #1
1690 GOTO 1410
1700 END



Author Cramer Michael Denis

Name of thesis The Short Term Effects Of The Supply Of Inorganic Nitrogen On The Photosynthetic Assimilation Of Carbon Dioxide. 1987

PUBLISHER:

University of the Witwatersrand, Johannesburg

©2013

LEGAL NOTICES:

Copyright Notice: All materials on the University of the Witwatersrand, Johannesburg Library website are protected by South African copyright law and may not be distributed, transmitted, displayed, or otherwise published in any format, without the prior written permission of the copyright owner.

Disclaimer and Terms of Use: Provided that you maintain all copyright and other notices contained therein, you may download material (one machine readable copy and one print copy per page) for your personal and/or educational non-commercial use only.

The University of the Witwatersrand, Johannesburg, is not responsible for any errors or omissions and excludes any and all liability for any errors in or omissions from the information on the Library website.