THE PATHWAY OF NITROGEN ASSIMILATION IN *DATURA STRAMONIUM* L.

by

Trevor A. Probyn

submitted in part fulfilment of the
requirements for the degree

MASTER OF SCIENCE

in the

Department of Botany,
Faculty of Science
University of Cape Town

June, 1978

CONTENTS

ACKNOWLEDGEMENTS

I wish to express my sincere appreciation to Professor O.A.M. Lewis, my supervisor, for suggesting the line of study and for his very helpful criticism, advice and guidance.

Mr. P. Haxen, B.Sc., performed the amino acid analyses and rendered valuable assistance in many other ways, for which I am most grateful.

Thanks also are extended to Mr. H. Botha and the departmental horticulturist, Mr. E. A. Smiltneek, for their help with the cultivation of experimental material.

I gratefully acknowledge the financial assistance of the C.S.I.R.

A special word of thanks to my typist Ms. K. S. Turk.

ABBREVIATIONS AND SYMBOLS

ALA	alanine
ARG	arginine
ASN	asparagine
ASP	aspartate
ATP	adenosine triphosphate
GDH	glutamate dehydrogenase
GLN	glutamine
GLU	glutamate
GLY	glycine
GOAT	glutamate-oxaloacetate aminotransferase
GOGAT	glutamate synthase
GPAT	glutamate-pyruvate aminotransferase
GS	glutamine synthetase
HIS	histidine
ILE	isoleucine
LEU	leucine
LYS	lysine
MET	methionine
^{15}N	nitrogen - 15
NAD	nicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate
NR	nitrate reductase
PHE	phenylalanine
SER	serine
THR	threonine
TYR	tyrosine
TRIS	tris (hydroxymethyl) aminomethane
VAL	valine

ABSTRACT

A survey of the recent literature concerning the assimilation of nitrogen into plant metabolism has been presented.

The pathway of nitrate-N assimilation into amino compounds by the leaves and roots of Datura stramonium at different nitrate feeding levels has been investigated using ^{15}N tracer experimentation, enzyme inhibitor studies and enzymological assays.

Leaves were fed via their xylem stream with potassium nitrate at two concentrational levels: 200 μg N. ml^{-1} and 25 μg N. ml^{-1}, prior to experimentation. Nitrate-^{15}N xylem stream and infiltration feeding experiments on Datura leaves indicate an apparent major routing of newly-reduced ^{15}N to glutamine at the high feeding level (200 μg N. ml^{-1}) and to glutamate at the low feeding level (25 μg N. ml^{-1}). Of the other major soluble amino compounds, serine, glycine, aspartate and alanine were found to be important in the primary assimilation of newly-reduced nitrogen.

A pretreatment of the leaves with 5 mM methionine sulphoximine, prior to nitrate-^{15}N infiltration, completely suppressed nitrogen assimilation into amino compounds with the resultant accumulation of ^{15}N in a large ammonia pool. Methionine sulphoximine also caused marked concentrational changes in the free amino compound pools, suggesting that conditions of nitrogen stress had been induced. Glutamate dehydrogenase activity (NAD(P)H-dependent) was not inhibited by the methionine sulphoximine pretreatment. Xylem stream feeding of glutamine-^{15}N at two concentrational levels (200 μg ^{15}N. ml^{-1} and

25 μg ^{15}N. ml^{-1}) revealed glutamate as the major acceptor of the amide-N of glutamine.

These results demonstrate that the assimilation of ammonia into amino compounds in Datura leaves was probably exclusively via the glutamine synthetase/glutamate synthase pathway, irrespective of nitrogen availability. The pattern of ^{15}N incorporation into glutamine and glutamate in response to feeding level can be explained by the snythesis of a large, storage glutamine pool outside the chloroplast during the pretreatment period at the high feeding level. This pool, being unlabelled with ^{15}N, serves to mask the activity of a smaller, actively turning-over pool of glutamine located in the chloroplast and synthesised from newly-reduced ^{15}N.

Enzymological assays on crude extracts of Datura leaves showed glutamine synthetase to be unaffected by the nitrogen concentrational difference between the two nitrate feeding levels employed in the pretreatment period. Nitrate reductase activity at the higher feeding level, however, was four times that at the low feeding level.

Nitrate-^{15}N infiltration experiments with roots established that they were capable of reduced nitrate-N assimilation into the soluble amino compound fraction, although at a much lower rate than in the leaves. Glutamine was by far the most heavily ^{15}N-labelled soluble amino compound. The effect of a pretreatment of the roots with 7 mM methionine sulphoximine suggested that in the roots, as in the leaves, the glutamine synthetase/glutamate synthase pathway was the sole route for 2-amino production from newly-reduced nitrogen.

CHAPTER 1

AIMS

The aim of this thesis was to investigate the pathway of newly-reduced nitrogen assimilation in both the leaves and the roots of the nitrophile Datura stramonium L. using ^{15}N-feeding experiments, enzyme inhibitor studies and enzymological assays. Experiments with leaves were performed on material pretreated with two concentrational levels of nitrate, to establish the effect, if any, of nitrate availability on the pathway of ammonia assimilation. Nitrate nitrogen was used in preference to ammonia for a number of reasons :

(a) Nitrate is the form of nitrogen most freely available to the roots of the majority of plants.

(b) In Datura nitrate accounts for more than 80% of the nitrogen reaching the leaves (Section 4.1.).

(c) It was felt unwise to bypass the nitrate/nitrite reduction stages because the utilization of nitrate after uptake may constitute a regulatory activity important in the normal physiological behaviour of a plant.

CHAPTER 2

INTRODUCTION

Although nitrogen constitutes a comparatively small fraction of the elements in the earth's crust (less than 0,005% by weight), its comparative abundance in the atmosphere (78.1% on a molar basis) and its ubiquitous and essential role in the biosphere make its metabolism of extreme interest to the plant physiologist. Ammonia made available by biological and non-biological means is oxidized to nitrate in the soil because the energy yield of the nitrification reaction (83,25 kcal.mole^{-1}) supports microbial activity (Delwiche, 1977). It is principally in this highly oxidized form (NO_3^-) that nitrogen is made available to higher plants (Hewitt et al., 1976). Nitrate once absorbed must be converted to its fully reduced form, the ammonium ion (NH_4^+), in order to make it available for organic synthesis. The enzymes of nitrate reduction, nitrate reductase and nitrite reductase, are now well-documented and the mechamism relatively well-established (see Reviews by Beevers and Hageman, 1969; Hewitt et al., 1976). The mechanism of ammonia assimilation, representing the ultimate step in the conversion of inorganic to organic nitrogen is, however, less clearly understood and demands attention, even beyond its physiological significance to the plant.

2.0 Minor Pathways of Ammonia Assimilation

Several reactions involving the direct assimilation of ammonia into organic union have been described in plants and micro-organisms. These include the reductive amination of 2-oxo acids, the formation of

the amides of aspartate and glutamate and the synthesis of carbamyl phosphate. A biosynthetic role has been assigned to aspartase (aspartate ammonia lyase) catalysing the direct amination of fumarate (Vender and Rickenberg, 1964) and alanine dehydrogenase (Germano and Anderson, 1968; Johansson and Gest, 1976) catalysing the reductive amination of pyruvate in bacteria and fungi. The essential physiological role for both enzymes, however, is now assumed to be degredative, producing carbon skeletons for energy metabolism (Meers et al., 1974). Alanine dehydrogenase is, however, thought to be assimilatory in a number of blue-green algae (Neilson & Doudoroff, 1973). Aspartate dehydrogenase (Santarius and Stocking, 1969) and alanine dehydrogenase (Joy, 1969) activities have been reported in plant cells though the enzyme has yet to be purified and characterised (Bryan, 1976). Kinetic studies utilizing the heavy isotope of nitrogen (^{15}N) do not, however, support a role for them in the primary assimilation of ammonia (Miflin and Lea, 1976). The synthesis of the amide asparagine via the amidation of aspartate has been demonstrated in micro-organisms but the enzyme catalysing the reaction, asparagine synthetase, is thought to serve a different function in plant cells, catalysing the ATP-dependent formation of asparagine and glutamate from glutamine and aspartate (Lea and Fowden, 1975a).

The synthesis of carbamyl phosphate, an intermediate in the synthesis of arginine and pyrimidines, is thought to involve glutamine, not ammonia, as a substrate and it is doubtful if the enzyme, carbamyl phosphate synthetase, contributes to ammonia assimilation in vivo (Brown et al., 1974).

2.1 Glutamate Dehydrogenase

It has been assumed, mainly on the basis of the careful $^{15}NH_4$ kinetic experiments of Sims and Foulkes (1964) using the yeast Candida utilis, and of Bassham and Kirk (1964a) using Chlorella, that the synthesis of glutamate by the reductive amination of 2-oxoglutarate, catalysed by glutamate dehydrogenase L-glutamate : NAD^+ oxidoreductase (deaminating) EC 1.4.1.2 is the mechanism of primary ammonia ssimilation in plant cells (Reaction 1) :

2-oxoglutarate + NH_4^+ + NAD(P)H ⟶ L - glutamate + H_2O + NAD(P) ...(1)

The reaction is physiologically reversible and can constitute an important catabolic process (Bryan, 1976). Besides being a method of ammonia assimilation, the reaction also serves as a link between carbohydrate and energy metabolism, and nitrogen metabolism. Despite the considerable physiological interest of the enzyme, the fundamental information about glutamate dehydrogenase (GDH) in higher plants is scanty compared to that in animals and micro-organisms. Purification and characterization have been carried out in only a few instances (Pahlich and Joy, 1971; Davies and Teixeira, 1975; Garland and Denis, 1977; Fawole and Boulter, 1977). The molecular weight of the pea root enzyme is 208000 (Pahlich and Joy, 1971) and that of pea seedling mitochondria, 230000 (Davies and Teixeira, 1975). The figures are similar to the figure of 250000 reported for the bacterium Mycoplasma laidlawii (Frieden, 1965) but a good deal smaller than the 320000 for Neurospora crassa (Grover and Kapoor, 1973).

It was widely believed that higher plants have only one type of GDH specific for NAD localized in the mitochondria (Ritenour et al., 1970;

Saigusa, 1970; Gayler and Morgan, 1976). Some authors propose the existence of distinct NAD and NADP specific GDH's (Grimes and Fottrell, 1966; Le John, 1971; Hwang and Soulen, 1972; Brown and Haslett, 1972) whilst others suggest a single GDH using unspecifically both co-enzymes (Yue, 1969; Gasparikova et al., 1976; Emmke and Hartman, 1976; Fawole and Boulter, 1977). Weissman (1972) has reported that the mode of nutrition affects the relative activity of the two forms of GDH in sunflower and soybean roots, ammonium-grown plants showing only NADH-GDH activity while nitrate-grown plants show both NADH and NADPH specific activity. Where these two forms of GDH are present within a single fungal species the NADP-linked enzyme acts in an anabolic role (Sanwal and Lata, 1961; Le John et al., 1968) while the main function of the NAD-linked enzyme is postulated to be the generation of NADH and in turn ATP with the concomitant breakdown of glutamate (Le John et al., 1968). Sanwal (1961), working with cultures of Fusarium, found the NADP-linked enzyme had the lower Michaelis constant (Km value) for ammonia while the NAD-linked enzyme had the lower value for glutamate. This, together with the evidence regarding the coordinate regulation of the anabolic and catabolic roles of GDH by glutamate (Westphal and Holzer, 1964; Sanwal and Lata, 1971), supports the proposal that in fungi the NAD - and NAD(P) - linked enzymes act in catabolic and anabolic roles respectively. This broad generalization has been made for most but not all fungi and bacteria (Brown et al., 1974) and has phyllogenetic implications when one considers that the lower fungi, Myxomycetes and Phycomycetes, possess only a NAD-specific enzyme while the Deuteromycetes, Ascomycetes and Basidiomycetes appear

to have both activities (Le John, 1971).

The isoenzymes of GDH are well documented (Thuruman et al., 1965; Chou and Splittstoeser, 1972; Kanamori et al., 1972). Although there are claims of separate NAD- and NADP-specific GDH's (possibly isoenzymes) located in different cell organelles (Leech and Kirk, 1968; Joy, 1969; Tsenova, 1972; Lea and Thuruman, 1972) and different organs of a plant (Sims et al., 1968; Marachal et al., 1977) there has been no case where two distinct GDH proteins have been separated from higher plants as has been done with fungi (Sanwal, 1961; Sanwal and Lata, 1961; Dennen and Niederpruem, 1967) and some bacteria (Le John and McCrea, 1968; Kramer, 1970). Lemna minor (Stewart and Rhodes, 1977) and pea roots (Pahlich and Joy, 1970) in fact have been shown to contain only a single molecular species corresponding to GDH activity.

As mentioned earlier, the GDH catalysed reaction is freely reversible, so a consideration of the Michaelis constants of the substrates and co-enzyme specificity might be indicative of the favoured reaction direction. Kanamori et al., (1972) found a much greater activity with NADH and NADPH (aminating) than with NAD^+ (deaminating) in rice roots. Similarly, Fawole and Boulter (1977) demonstrated a 126-fold greater NADH-specific activity than NAD^+ activity in Vigna unguiculata mitochondria. In the pea the ratio Vmax NADH oxidation/Vmax NAD^+ reduction is 25 for both the mitochondrial and soluble enzyme preparations (Davies and Teixeira, 1975). It appears then that mitochondrial GDH of higher plants, believed to be mainly NAD-specific, acts in an anabolic role catalysing the synthesis of glutamate. Pahlich and Joy (1970), however, working on a highly

purified pea root GDH have shown that the ratio of NADH (aminating) to NAD^+ (deaminating) activities is not constant, being markedly affected by the extraction buffer used (tris or phosphate) and the presence of calcium ions. The *in vivo* role of mitochondrial GDH thus remains obscure though work on *Lemna* (Joy, 1969; Shepard and Thuruman, 1973) has shown that the NAD-linked GDH may have an assimilatory role.

Bassham and Kirk (1964) in their pioneering studies on *Chlorella* thought the chloroplast to be intimately involved in the primary assimilation of nitrogen. Nitrite reductase is known to be localized entirely within the chloroplast (Ritenour *et al.*, 1967; Dalling *et al.*, 1972) and it would seem logical that the enzymes of ammonia assimilation would be similarly located. The demonstration of a light dependent reduction of nitrite to 2-amino nitrogen by isolated spinach chloroplasts (Magalhaes *et al.*, 1974) provide confirmation of this hypothesis.

If this light dependent reduction is mediated by GDH it is an obvious conclusion that the enzyme must be located in the chloroplast. Workers following on the early lead of Bassham and Kirk (1964) have detected a chloroplast GDH and the ability of intact chloroplasts to reduce 2-oxoglutarate to glutamate (Leech and Kirk, 1968; Givan *et al.*, 1970). It has been speculated that photosynthetically reduced pyridine nucleotide might be used in the reductive amination to glutamate (Bassham and Jensen, 1967), a view supported by the fact that the outer envelopes of intact chloroplasts are quite impermeable to pyridine nucleotides (Heber and Santarius, 1965; Robinson and Stocking, 1969; Heber, 1974). The general stimulatory effect of light on GDH activity (Brown and Haslett, 1972; Welander, 1974) supports the view

that products of photosynthesis might be involved. The demonstration of an NADP-dependent GDH in chloroplasts (Leech and Kirk, 1968; Lea and Thuruman, 1972) and the regulation of the reductive amination of 2-oxoglutarate by the availability of NADPH and light intensity (Givan et al., 1970) suggest that the GDH of chloroplasts is NADP-specific and that the NADPH is partly if not wholly photosynthetically produced. Rantham and Edwards (1976) have shown that in Panicum 70-77% of the total NADPH-dependent activity is localized in the chloroplast fraction with only 18-22% of the NADH-activity being similarly located. In addition to assimilatory GDH, chloroplasts have been shown to possess the necessary amino-transferases for the production of aspartate and alanine from glutamate (Santarius and Stocking, 1969). Working on Vicia faba, Kirk and Leech (1972) have shown that all common protein amino acids with the exception of leucine could be synthesized by intact chloroplasts in secondary amino-transferase reactions from alanine or from aspartate. The GDH pathway then has the definite potential for the primary assimilation of ammonium by chloroplasts.

The recorded rates of photoreduction of 2-oxoglutarate by isolated chloroplasts, however, are low, being 0,4 µmole.mg $Chl.^{-1}hr^{-1}$ for spinach (Tsukamoto, 1970) and 0,6 µmole.mg $Chl.^{-1}hr^{-1}$ for the broad bean (Givan et al., 1970). These values are scarcely sufficient to account for the rates of light dependent 2-amino nitrogen production by intact chloroplasts of 9,0 µmole.mg $Chl.^{-1}hr^{-1}$ (Miflin, 1974) and 12,0 µmole. mg $Chl.^{-1}hr^{-1}$ (Magalhaes et al., 1974). The low amounts of GDH isolated from chloroplasts may be due to the difficulty in solubilizing the enzyme from chloroplast lamellae (Lea and Thuruman, 1972).

The disparity, however, is likely to be more marked *in vivo* as a result of the high Km for ammonia of GDH. This low affinity of GDH for ammonia is the major limitation to the functioning of this pathway in higher plant chloroplasts. Some exceptionally high Michaelis constants for ammonia include : Km = 10.1mM for corn (Bulen, 1956); Km = 172mM for *Phaseolus aurens* (Lea and Thuruman, 1972); and Km = 70mM for pea epicotyls (Davies and Teixeira, 1975). The only exception amongst higher plants appears to be a NAD specific GDH in peas where the exceedingly low apparent Km for ammonium and 2-oxoglutarate of 42 and 2,9 uM respectively have been reported (Tsenova, 1972). Seeing that ammonium concentrations as low as 0,5 - 5mM cause markedly decreased rates of ATP production by chloroplasts (Avron, 1960), a result of the uncoupling of electron transport (Good, 1960), it is unlikely that chloroplastic ammonium concentrations could rise to the levels necessary for the realization of the assimilatory GDH potential. An exception appears to be the marine diatom *Ditylum brightwellii* which possess the ability to accumulate ammonia possibly to aid GDH activity (Eppley and Rogers, 1970), the Km value for ammonia being about 10mM (Brown *et al.*, 1974) while the internal ammonia concentration reaches level of 5-10 mM (Eppley and Rogers, 1970). The algae actually appear to be a group of plants that possess a GDH with suitable characteristics for assimilation of ammonia. The green alga *Chlorella* vulgaris has a low Km for ammonia, being in the region 0,3 to 0,5 mM (Brown *et al.*, 1974; Lea and Miflin, 1975 unpublished results). The activity, too, of the *Chlorella* enzyme is sufficient to be acting in an assimilatory role, being about three times that required for ammonia assimilation (Morris and Syret, 1965).

Caulerpa simpliciuscula, another green alga, has a similarly low Km for ammonia of 0,67 mM, at least an order of magnitude lower than those of higher plants. The enzyme is NADP-dependent and chloroplastic which makes for a direct economical route to glutamate (Gayler and Morgan, 1976).

The strength of this argument regarding the participation or non-participation of GDH in ammonia assimilation based on the Michaelis constants for ammonium, is weakened by the fact that the Km's of purified enzymes may be radically different from their in vivo values (Eg, Bahr and Jensen, 1974) or that the enzyme is compartmentalized in close proximity to nitrite reductase within the chloroplast (Miflin and Lea, 1976).

2.2 Glutamine Synthetase

Another reason (apart from Km values) for doubting a dominant role for GDH in ammonia assimilation in chloroplasts is the presence of glutamine synthetase (GS) (EC.6.3.1.2) in chloroplasts (O'Neal and Joy, 1973; Haystead, 1973). Glutamine synthetase catalizes the irreversible amidation of glutamate and is ATP dependent (Reaction 2) :

$$\text{Glutamate} + NH_4^+ + \text{ATP} \longrightarrow \text{Glutamine} + \text{ADP} + \text{iP} \quad \ldots\ldots(2)$$

Calculations based on the distribution of chloroplast marker enzymes suggest that a minimum of 32% and probably more than 66% of higher plant GS is located in the chloroplast (O'Neal and Joy, 1973a). There is no evidence for the presence of this enzyme in any other organelle, but it is probably present in the cytoplasm (Miflin, 1974a). Glutamine synthetase activities (e.g. 90 μmole.mg $Chl.^{-1}hr^{-1}$ in spinach leaves (Miflin, 1974a) being several times that of GDH, are more than sufficient to cope with the reported rates of amino nitrogen production,

e.g. 12 μmole.mg $Chl.^{-1}hr^{-1}$ (Magalhaes et al., 1974) by isolated intact spinach chloroplasts. In addition it has a low Km for ammonia : Km = 0,3 mM in peas (Varner, 1960); Km = 0,67 mM in pumpkin seeds (Lignowski et al., 1971); 0,4 mM in rice roots (Kanamori and Matsumoto, 1972) and 15 μM in Lemna minor (Stewart and Rhodes, 1977). It is unlikely then that GDH could compete successfully with GS for ammonia within the chloroplast. Illuminated chloroplasts will convert glutamate to glutamine at a rate of about 10 μmoles.mg $Chl.^{-1}hr^{-1}$, roughly comparable with the measured rates of nitrite reduction to ammonia (Miflin, 1974b).

The light dependence of the process suggests that ATP generated by photophosphorylation is used in the GS catalysed reaction. Considerable evidence is consistent with the operation of a cyclic photophosphorylation process entirely dependent on photosystem I (Simonis and Urbach, 1973). On the other hand, Givan (1975, 1976) has suggested a major role for non-cyclic or pseudocyclic (requiring both photosystems, the latter having an O_2 requirement as well) phosphorylation in glutamine synthesis by chloroplasts. Mitchell and Stocking (1975), however, support Simonis and Urbach's view that cyclically generated ATP could support glutamine synthesis. There is no evidence yet available, however, to suggest that ATP generated by any one specific type of phosphorylation is inherently required to drive glutamine synthesis (Mitchell and Stocking, 1975; Givan, 1976).

Glutamine synthetase has been purified from pea leaves (O'Neal and Joy, 1973, 1974) and has been found to have a molecular weight of 330000 - 370000, similar to the pea seed enzyme (Tate and Meister, 1971)

but smaller than the 592000 recorded for E. coli (Shapiro and Ginsburg, 1968). An even lower molecular weight has been recorded for the carrot cell GS which is estimated to consist of two fractions of molecular weights 180000 and 56000 - 79000 (Caldos, 1971 quoted by O'Neal and Joy, 1973). A two-layered structure has also been reported for the E. coli enzyme (Valentine et al., 1968) and appears to be a common feature of all glutamine synthetases (Miflin and Lea, 1977). One of the most unusual properties of the pea leaf GS is the effect of divalent cation species on the substrate specificity and kinetic behaviour of the enzyme. The pH optimum varies between 5.2 and 8.3 depending on the divalent cation present and their concentrations (O'Neal and Joy, 1973). The three most effective cations for the pea enzyme are Mn^{2+}, Mg^{2+} and Co^{2+} as is the case with most bacterial and animal glutamine synthetases (Monder and Jacobson, 1964; Hunt and Ginsburg, 1972). In the pea leaf Mn^{2+} results in a low specificity for nucleotide tri-phosphate similar to the situation in Bacillus subtillis (Deuel and Stadtman, 1970). Magnesium ions gave optimal activity in the pea leaf similar to the carrot enzyme (Caldos, 1971 quoted by O'Neal and Joy, 1974) but differs from the pea seed enzyme where Co^{2+} is the most effective divalent cation (Tate and Meister, 1971). The relative physiological significance of these divalent cation effects in vivo, however, remains to be established (Miflin and Lea, 1977).

2.3 Discovery of Glutamate Synthase

If GS were to act as an ammonia assimilatory (and not merely detoxifying) enzyme and hence glutamine were to lie on the pathway of amino acid synthesis from ammonia, then organisms would need some enzyme or enzyme system capable of transferring the amide nitrogen of glutamine to the 2-amino position. The pioneering work on such a system was done with bacteria by Tempest and his colleagues in Wiltshire. It is relevant at this stage to consider briefly the assimilation of ammonia by bacteria. The presence of GDH in bacteria is well established (Meers et al., 1970a; Meers and Kjaergaard-Pedersen, 1972) and it has been assumed to be mainly biosynthetic in its role. Taking into account the high Km for ammonia of bacterial GDH, approximately 4mM (Miflin and Lea, 1976), it is unlikely that it functions efficiently except when the environmental ammonium concentration is high (Brown et al., 1974). For example, chemostat cultures of Aerobacter aerogenes growing with glucose and orthophosphate as limiting substrate (and in the presence of excess ammonia) synthesized appreciable concentrations of NADP-linked GDH (Meers, Tempest and Brown, 1970a). In ammonia limited cultures, however, when the intracellular ammonium level was less than one-tenth the Km for ammonia, the GDH content fell to about 3% of its original level and therefore could not adequately fill a biosynthetic role. Adding a pulse of ammonia to steady state ammonia-limited cultures caused a rapid 25-fold increase in the intracellular glutamine pool and also resulted in an 80-fold increase in the GS level over that present in an excess of ammonia (Tempest et al., 1970). This indicated

that ammonia assimilation under these conditions proceeded via the amidation of glutamate but no enzyme was yet known that could catalyse the transfer of the amide nitrogen of glutamine into the 2-amino position of glutamate. Tempest et al. (1970), however, were able to describe such an enzyme capable of the reductive transfer of the amido group of glutamine to 2-oxoglutarate to give two molecules of glutamate. The co-factor required was NADPH and the enzyme was classified as E.C.2.6.1.53 glutamine : 2 oxoglutarate aminotransferase (NADPH oxidizing) (Reaction 3) :

Glutamine + 2-oxoglutarate + NADPH $\longrightarrow$ 2 glutamate + $NADP^+$..... (3)

The enzyme has been reclassified by the International Union of Biochemistry as E.C.1.4.1.13 glutamate : $NADP^+$ oxoreductase (transaminating). The reaction is undirectional (Nagatini et al., 1971; Brown et al., 1972) and the enzyme has been given the trivial name 'glutamate synthase' and the acronym 'GOGAT'.

The synthesis of glutamate via GOGAT requires the participation of an active GS. Significantly bacterial glutamine synthetases are found in considerable quantities in organisms where growth is limited by ammonia deprivation (Woolfolk et al., 1966; Wu and Yuan, 1968; Pateman, 1969). The Km of bacterial synthetases for ammonia, like that of higher plants, is usually low; values of 1,0mM for Aerobacter aerogenes (Tempest et al., 1970) and 0,2mM for E.coli (Denton & Ginsburg, 1970) have been reported.

The end result of the GS/GOGAT pathway and the one mediated by GDH is the same, the major difference, however, is that the synthesis of glutamine requires the expenditure of energy in the form of ATP.

Presumably this energy expenditure is the "price that organisms pay" to assimilate low concentrations of ammonia (Tempest et al., 1970). The first suggestion of such a mechanism was made by Umbarger (1969) who proposed that under conditions of nitrogen limitation, the ATP-driven conversion of ammonia to an amide group acted as a "pump" to scavenge the last traces of ammonia from the environment. Since the first report of this pathway it has been shown to operate in many other bacterial species (Elmerich and Aubert, 1971; Berbereich, 1972; Brown et al., 1972). Glutamine synthetase has also been implicated in the immediate assimilation of ammonia produced by symbiotic nitrogen fixation (Kennedy, 1966a, 1966b). Both GS and GOGAT have been found in the bacteriods of most nitrogen-fixing associations. The levels of GOGAT are, however, only sufficient to deal with 10-20% of the nitrogen flux (Miflin and Lea, 1976). Further work is required to confirm the flow of nitrogen in nodules. Free living nitrogen fixing bacteria contain GS and GOGAT (Nagatini et al., 1971) as does the blue-green alga Anabaena cylindrica (Thomas et al., 1975; Rowell et al., 1977). In vivo tracer studies with ^{14}C in Anabaena show a rapid accumulation of label in glutamine on addition of ammonium or nitrogen gas (Lawne et al., 1976). It appears, then, that the GS/GOGAT pathway is involved in the assimilation of ammonia produced by the reduction of N_2 by nitrogen fixing organisms.

Glutamate synthase has been purified to homogeneity from E.coli (Miller and Stadtman, 1972; Miller, 1974). The available data suggest that GOGAT might be composed of eight sub-units, four of each of two types of dissimilar sub-units of molecular weights 135000 and 53000

aggregated to give a final molecular weight of 800000. In addition, each molecule probably contains 32 iron atoms, 32 labile sulfide atoms and eight covalently bound flavin molecules. The GOGAT mediated reaction appears to be the sum of two partial reactions. The first step involves the NADPH-dependent reduction of the enzyme-bound flavin, perhaps involving iron and sulfide. The second step involves the conversion of 2-oxoglutarate and L-glutamine to glutamate. The enzyme is highly specific for glutamine and 2-oxoglutarate; Km's of 250 μM and 7,3 μM respectively (Miller and Stadtman, 1972). Other Km values include 0,6-5,0mM for glutamine (Brown et al., 1972) and 0,2mM for both glutamine and 2-oxoglutarate (Dainty, 1972). Meers et al., (1970b) and Meers and Kjaergaard-Pedersen (1972) found the enzyme from a variety of organisms was only active with NADPH as an electron donor. Nagatini et al., (1971) working with Aerobacter pneumoniae, and Brown et al., (1972) working on a number of marine bacteria, found some species that synthesized a NADH-specific enzyme. Dual co-enzyme specificity is reported for Clostridium pasteurianum (Dainty, 1972) but no organism has yet been found that has both NADPH- and NADH-linked enzymes.

The first demonstration of analogous GOGAT activity in higher plants was in extracts of carrot cell cultures by Dougall (1974). He discovered a GOGAT that was active with NADPH or NADH. A similar lack of reduced pyridine specificity has been reported for sycamore and pea root cells (Fowler et al., 1974). Beevers and Storey (1976) demonstrated NADPH- and NADH-specific GOGAT activities in developing pea cotyledons, though the rate of oxidation of NADH was higher (Michaelis constants of 13,3 μM for NADH and 27,7 μM for NADPH). It has been suggested

that the requirement could actually be for NADH as is the case in lupin roots (Robertson et al., 1975), with a pyridine nucleotide phosphatase converting NADPH to NADH, the preferred electron donor (Beevers and Storey, 1976). The NADH- and NADPH-dependent activities are thought to be due to two different enzymes rather than a single enzyme using both co-enzymes unspecifically (Dougall and Bloch, 1976).

Shortly after the discovery of GOGAT in carrot cells (Dougall, 1974), Lea and Miflin (1974) detected a GOGAT-type activity in green tissue in response to a previous report by Lewis and Pate (1973). The latter authors found that in Pisum sativum the ^{15}N of three ^{15}N-labelled substrates, nitrate, glutamate and glutamine (amide ^{15}N), were incorporated in the same relative manner into all amino compounds. Were a GOGAT-type activity not operative in this case the amide group of glutamine would only donate its label to tryptophan, arginine and histidine (Miflin and Lea, 1976). Following this lead, Lea and Miflin (1974), using the same plant material as Lewis and Pate (1973), were able to describe GOGAT activity in the leaves of a higher plant. They found that addition of glutamine to isolated chloroplasts of Pisum caused a marked stimulation of glutamate formation and that this effect was light dependent. Ammonia did not stimulate this reaction, ruling out the participation of GDH. Spectrophotometric assays of chloroplast extracts demonstrated the presence of a 2-oxoglutarate and glutamine dependent synthesis of glutamate. Ferredoxin was an extremely effective electron donor and no NAD(P)H-dependent activity could be detected, consistent with other studies (Haystead, 1973; Rathnam and Edwards, 1976; Duke and Koukkari, 1977). Lea and Miflin (1974) thus

proposed a similar system for higher plants to that demonstrated in bacteria (Tempest et al., 1970) whereby glutamate synthase catalysed the formation of two molecules of glutamate from glutamine and 2-oxoglutarate, the major differences being the light dependence (Nicklisch et al., 1977) and the participation of ferredoxin and not a pyridine nucleotide. A ferredoxin-dependent GOGAT is also active in wheat (Nicklisch et al., 1976) and Lemna minor (Rhodes, et al., 1976). The enzyme shows an exceedingly high affinity for ferredoxin, values as low as 2 μM having been reported (Wallsgrove et al., 1977). A dithionite-dependent GOGAT is present in the unicellular green alga Chlorella (Lea and Miflin, 1975). The dithionite presumably acts to reduce the internal ferredoxin. It appears that the GOGAT of green tissue is ferredoxin-dependent while that of non-photosynthetic tissue has a reduced pyridine nucleotide requirement. Marechal et al. (1977), have shown the GOGAT of Phaseolus and Oryza roots to be NADPH-dependent and inactive with ferredoxin while the leaf tissue from the same plants exhibited only a ferredoxin-dependent GOGAT. As with GS, higher plant GOGAT is located primarily in the chloroplast, some 84% of the total activity being recorded in Panicum chloroplasts (Rantham and Edwards, 1976). The enzyme has been purified from bean leaves by Wallsgrove et al. (1977). Higher plant GOGAT appears to be different to the bacterial enzyme (Miller and Stadtman, 1972) in that no non-haem and flavin component could be detected. The molecular weight of 145000 is also considerably below that of 800000 recorded for E.coli (Miller and Stadtman, 1974). The active site of glutamate synthase appears to be highly specific for L-glutamine, being inactive

with purified asparagine (Miflin and Lea, 1975) and 12 glutamine analogues (Wallsgrove et al., 1977). The Km values for glutamine are low: 0,32 mM for bean leaves (Wallsgrove et al., 1977), 1,43 mM for pea cotyledons (Beevers and Storey, 1976), and 0,6 mM for pea chloroplasts (Anderson and Done, 1977). Measured Km values for 2-oxoglutarate of 0,96 mM (Beevers and Storey, 1976), 0,15 mM (Wallsgrove et al., 1977) and 0,2 mM (Anderson and Done, 1977) indicate a high affinity. Other 2-oxoacids tested for their ability to act as acceptors of the amide group of glutamine were found to be inactive (Wallsgrove et al., 1977).

2.4 Additional Evidence Favouring the GS/GOGAT Pathway

The previous section dealing mostly with the characteristics and distribution of the relevant enzymes suggest that in photosynthetic organisms or tissues, ammonia is assimilated first into the amide position of glutamine and then transferred into the 2-amino position of glutamate. However, it must be remembered that the demonstration of an enzyme in a cell established only the possibility that the reaction it catalyses may be metabolically functional. Conversely, the apparent lack or low activity of an enzyme must be viewed with caution because of the inherent difficulties of enzyme isolation and measurement (Bryan, 1976). More conclusive, physiologically relevant information is gained from kinetic experiments utilizing stable isotopes of nitrogen. Other than the studies of Sims and Foulkes (1964) and Bassham and Kirk (1964), most ^{15}N-feeding experiments indicate that glutamine, not glutamate, is the prime recipient of newly reduced ammonia in higher plants. The work of Yem and Willis

(1956) on excised shoots of barley suggested that the synthesis of glutamine was an important feature of primary ammonia assimilation. Barley seedlings showed a similar pattern of labelling with both the amino and amido nitrogen of glutamine becoming extensively labelled after one hour of ammonium-^{15}N treatment (Cocking and Yemm, 1960). Isotopically labelled ammonium has also been fed to rice seedlings (Yoneyama and Kumazawa, 1974; Arima and Kumazawa, 1975), sunflower leaves (Ito and Kumazawa, 1976) where again it was found that glutamine had the highest ^{15}N enrichment initially. The ^{15}N content of glutamate, aspartate and alanine followed glutamine in that order. A different pattern of labelling was revealed when nitrate ^{15}N was applied to sunflower leaves. The nitrate fed leaves had a lower total ^{15}N content to the ammonium fed leaves and in this case glutamine showed a lower enrichment than glutamate and aspartate. It was concluded that ammonium formed by the reduction of nitrate was assimilated by a pathway different from exogenously applied ammonium (Ito and Kumazawa, 1976). Experiments on excised shoots of Datura stramonium, however, have shown glutamine to be the main portal of ^{15}N entry after feeding for several hours with nitrate ^{15}N (Lewis, 1975; Lewis and Berry, 1975). Short-term experiments with nitrate ^{15}N, which have been performed on rice seedlings (Yoneyama and Kumazawa, 1975) and corn roots (Yoneyama et al., 1977), discount the claims of Ito and Kumazawa (1976) that newly reduced nitrogen is assimilated via a different pathway to exogenously supplied ammonium.

Many of these experiments can be criticised on the basis that they do not take the individual pool sizes of the amino acids and amides into account. Neglect of this parameter can be misleading

when one is considering the relative diversion of ^{15}N amongst the relevant amino acids and amides. For example, Leaf et al. (1959) found the amide nitrogen of glutamine to have the highest enrichment. However, because of the large pool size of asparagine, the actual diversion of ^{15}N to this amino acid was 3,5 times that of glutamine.

The use of amino acid analogues has provided another line of evidence favouring the existence of the GS/GOGAT pathway. L-Methionine sulphoximine (MSO) is an analogue of the glutamylphosphate enzyme complex of GS (Miflin and Lea, 1976) and a potent inhibitor of the enzyme (Ronzio et al., 1969; Ownby, 1977). Glutamate dehydrogenase is unaffected by this enzyme (Brenchley, 1973). Glutamate synthase is inhibited by the glutamine analogues 6 - diazo - 5 - ovo - L - norvaline (DON) and azaserine which block all glutamine-amide transfer reactions and do not affect GDH (Miller and Stadtman, 1972; Lea and Norris, 1976). Consequently, application of these analogues should inhibit nitrogen assimilation via the GS/GOGAT pathway but have no effect on GDH activity. Stewart and Rowell (1975) have shown that addition of MSO to N_2-fixing cultures of Anabaena cylindrica causes a decrease in the intracellular pool of glutamine and glutamate and an increase in the ammonia concentration. Working on the same organism, Wolk et al. (1976) showed that 1 mM methionine sulphoximine permitted the formation of ammonia from $^{13}N_2$ but prevented ^{13}N labelling of amino compounds. It was previously shown (Thomas et al., 1975) that glutamine is the first ^{13}N-labelled organic product observed and glutamate the second. Azaserine application stopped the labelling of all amino acids and amides except glutamine. These experiments demonstrate unequivocally that N_2 derived ammonia is assimilated by the combined action of GS

and GOGAT in the free-living Anabaena. Studies monitoring the response of relevant amino compound pool concentrations in Lemna minor to MSO and azaserine treatment suggest the operation of the GS/GOGAT pathway (Rhodes and Stewart, 1977). As of yet the use of these analogues in conjunction with ^{15}N-feeding experiments have not been attempted in higher plants.

2.5 The Regulation of the Major Ammonia Assimilating Enzymes

Available information on the regulation of the major ammonia assimilatory enzymes in micro-organisms far surpasses that on higher plants for which very little interpretation and synthesis of the accumulated data has been attempted. The presence of GDH in micro-organisms is well documented and the enzyme has been shown to be subject to rather intensive control (Pateman, 1969). The central position of glutamine in nitrogen metabolism, the amino and amide groups being utilized in diverse pathways (Meister, 1962; Hubbard and Stadtman, 1967), has necessitated the evolution of complex and unique mechanisms of control of GS. Mechanisms involving the inter-conversion between "relaxed" (inactive) and "taut" (active) forms of GS (Shapiro and Stadtman, 1967; Shapiro and Ginsburg, 1968) and the adenylating/de-adenylating system (Kingdon et al., 1967; Shapiro and Stadtman, 1968) demonstrated in E.coli, have not been shown to be operative in higher plants (Miflin and Lea, 1977; Kingdon, 1974 respectively). There have been claims of cumulative feedback inhibition of GS in Lemna minor (Rhodes and Stewart, 1974), pea leaves (O'Neal and Joy, 1975) and soybean (McFarland et al., 1976), but they have not been

substantiated as has been done for E.coli (Woolfolk and Stadtman, 1964, 1967). One method of control at the level of enzyme synthesis reported for the GS of E.coli (Mecke and Holzer, 1966) which has particular relevance to the present discussion is the repression and de-repression of GDH and GS biosynthesis in response to variations in the nitrogen supply.

The GDH pathway being essentially reversible is subject to mass action effects determined by the concentrations of the reactants involved. Ammonia greatly stimulates GDH activity, assumed to be biosynthetic, in both micro-organism (Sanwal and Lata, 1962; Pateman, 1969) and higher plants (Joy, 1969; Shepard and Thuruman, 1973) while glutamate inhibits the aminating reaction in Brevibacterium flavum (Shiio and Ozaki, 1970), Lemna minor (Stewart and Rhodes, 1977), and pea roots (Joy, 1973). On the other hand, growth of E.coli (Mecke and Holzer, 1966), Aerobacter aerogenes (Tempest et al., 1970) and Lactobacillus arabinosis (Ravel et al., 1975) on relatively high quantities of ammonium resulted in a low level of GS. Limiting quantities of ammonium in the culture medium, however, were found to de-repress GS synthesis some 80 fold (Tempest et al., 1970). A similar response is evident in the higher plant Lemna minor, where the activity of GS was lowest in plants grown on ammonia (and glutamine) and highest in those grown on nitrate (Rhodes et al., 1976). The fact that nitrate grown plants are characterized by low ammonia (and glutamine) pool sizes (Orebamjo and Stewart, 1974), suggests a similar situation exists in higher plants as in micro-organisms, whereby the intracellular ammonium concentration controls both GDH and GS synthesis. A marine pseudomonad, strain SW_2, with

200 $\mu g.ml^{-1}$ ammonium-N as the nitrogen source, showed complete repression of GS synthesis, and ammonia assimilation was carried out by GDH, while growth on 50 $ug.ml^{-1}$ ammonium-N or nitrate-N (independent of concentration) resulted in the GS/GOGAT pathway assuming the major ammonia assimilatory role (Brown et al., 1972). An inverse relationship between GDH and GS has been demonstrated in Lemna minor (Rhodes et al., 1976). The GDH/GS ratio increases as a function of the external nitrate and ammonium concentrations. Glutamate synthase parallels GS in response to increasing nitrate and ammonium concentrations in Lemna (Rhodes et al., 1976). The reduction in GS levels as a result of increasing ammonium concentrations has been shown to be an indirect response in both Lemna minor (Rhodes et al., 1975) and the yeast Candida utilis (Ferguson and Sims, 1974), the intracellular glutamine pool being the actual effector. This situation differs in bacteria where it is the ammonium concentration solely which controls GS activity (Meers and Tempest, 1970; Brown et al., 1972).

The proposed increasing importance of the GDH pathway in ammonia assimilation with increasing external nitrate and ammonium concentrations in the higher plant, Lemna minor (Stewart and Rhodes, 1977), can be criticized on the grounds that the changes in enzyme levels are not great. Miflin and Lea (1977) are of the opinion that the residual GS activity may be more than sufficient to account for ammonia assimilation under conditions favouring the GDH pathway. This hypothesis has been substantiated in Lemna where the GS/GOGAT pathway has the potential to remain the major route for ammonia assimilation irrespective of ammonia availability (Rhodes and Stewart, 1977).

2.6 Summary

The chloroplast is undoubtedly intimately involved in the process of ammonia assimilation, functioning not only as the location for the assimilatory enzymes but also supplying the required ATP and reducing energy for the GS/GOGAT and GDH pathways.

Results from ^{15}N studies, the effects of specific inhibitors and the regulation and distribution of the relevant enzymes all suggest that the assimilation of ammonia first into the amide group of glutamine and then into glutamate in photosynthetic organisms and tissues, provides an important alternative route of 2-amino production from ammonia. The crucial ^{15}N-feeding experiments in conjunction with MSO treatment, however, have not been performed on leaf tissue as of yet and the effect of nitrogen availability on the pathway of ammonia incorporation by higher plants requires clarification.

Available information on the mechanism of ammonia assimilation by non-symbiotically associated roots is scanty and demands further investigation.

CHAPTER 3

MATERIALS AND METHODS

3.1 Plant Material

Three to four week old plants of the thorn apple Datura stramonium L. were used for experimentation. Seeds were germinated on trays of vermiculite in a Conviron growth cabinet under conditions of 100% humidity and 25°C. It was found that allowing seeds to imbibe a dilute (0,1 $\mu g.ml^{-1}$) gibberellic acid solution overnight markedly increased the frequency of germination during the winter months. Datura, however, proved to be slow and unpredictable in its germination, even at the best of times.

Seedlings were potted out in vermiculite and transferred to a greenhouse where they were raised on a modified Hoagland solution containing 100 μg N. ml^{-1} nitrate. Details of the composition of all nutrient solutions used in this study are summarized in Tables 1 and 2. The nutrient solution was administered every second day. Datura was also grown hydroponically in 20 l asbestos pots, with continuous aeration, at 50, 100 and 200 μg N. ml^{-1} nitrate (Tables 1 and 2).

3.2 Pretreatment - Induction Period

Prior to experimentation whole potted plants were transferred to a Conviron growth cabinet and left for two days under conditions of a 14 hr photoperiod and a temperature of 21°C. Irradiance was supplied by Sylvannia (Canada) cool white fluorescent tubes (5,76 $mW.cm^{-2}$), supplemented with 60 W incandescent lamps (4,66 $mW.cm^{-2}$).

TABLE I

Amounts in grams of the macronutrients used to make up 10 l of nutrient solution at the three nitrate concentrations.

Macronutrients	50 µg N. ml^{-1}	100 µg N. ml^{-1}	200 µg N. ml^{-1}
KNO_3	3,61	7,22	14,44
K_2SO_4	4,70	1,58	-
$CaCl_2.6H_2O$	5,00	5,00	5,00
$MgSO_4$	2,48	2,48	2,48
$NaH_2PO_4.2H_2O$	2,84	2,84	2,84

TABLE 2

Amounts in milligrams of micronutrients used in 10 l of nutrient solution. The levels of micronutrients were kept the same for all nitrate feeding levels.

Micronutrients	
HBO_3	85,8
$MnSO_4.H_2O$	46,4
$ZnSO_4.7H_2O$	6,6
$CuSO_4.5H_2O$	2,4
H_2MoO_4	0,6
FeNaEDTA	330,0

The dark period temperature was 18°C and the relative humidity maintained at 70%. Because of the marked effect of temperature (Yoneyama et al., 1977) and irradiance (Tychsen, 1976) on amino acid metabolism, it was considered necessary to perform all experiments under these physical conditions.

At the commencement of an experiment, whole leaves were severed under water and fed through their petioles with distilled water containing either 25 $\mu g.ml^{-1}$ or 200 $\mu g.ml^{-1}$ nitrate -N for 4-6 hours. Experiments in this laboratory have shown that this time period is sufficient to fully induce nitrate reductase to its characteristic levels for both feeding levels. Longer periods proved unsatisfactory as there was a tremendous build-up of glutamine in the leaves in the absence of such natural 'sinks' as the roots and seeds.

In methionine sulphoximine (MSO)-treated leaves the induction period was divided into 4 hrs of ^{14}N-nitrate feeding, at the two concentrational levels, followed by 2 hrs of feeding a solution containing the ^{14}N-nitrate and 5 mM L-methionine-DL-sulphoximine (Oaks, pers. comm.).

Root tissue was pretreated at one concentration of nitrate, 100 μg N. ml^{-1}. After carefully washing the roots free of vermiculite, whole plants were transferred to a water culture solution (100 μg N. ml^{-1} nitrate) two days prior to experimentation. Only plants showing no visible sign of deterioration after transfer were used in subsequent experimentation. Roots to be treated with MSO were then placed in a complete nutrient solution containing 7 mM MSO for 3 hrs. It was found necessary to extend the period of MSO pretreatment and to raise the concentration of MSO used over that employed

for leaf tissue, in order to elevate root intracellular MSO to levels similar to those obtained in leaf tissue. Aeration in all cases was continuous.

3.3 Methods of Isotope Feeding

The ^{15}N-labelled substrates L-glutamine (amide ^{15}N, 97 atom percent ^{15}N) and potassium nitrate (99 atom percent ^{15}N) were fed in one of three ways :-

i) through the transpiration stream (Lewis and Pate, 1973)

ii) via vacuum infiltration (Ito and Kumazawa, 1976) and,

iii) via pressure infiltration

3.3.1 Transpirational Feeding

This method involved transferring whole leaves to solutions containing 200 μg ^{15}N. ml^{-1} or 25 μg ^{15}N. ml^{-1} as potassium nitrate or glutamine for varying lengths of time up to 45 mins. The feeding solution was taken up through the petiole in the transpiration stream.

This method has the advantage of preserving the structural integrity and intercellular environment of the leaf but the time lag between the application of the ^{15}N substrate and the actual time of uptake by leaf cells is a serious disadvantage, limiting the time course nature of such experiments. Perhaps even more important is the dilution effect by the intracellular unlabelled pools of nitrate in the leaf. Intracellular nitrate levels in Datura leaves are of the order of 100 μg N. gFW^{-1} (Table 3) whereas they were only receiving

approximately 18 μg ^{15}N. $gFW^{-1}.hr^{-1}$ and 10 μg ^{15}N. $gFW^{-1}.hr^{-1}$ from the 200 μg ^{15}N. ml^{-1} and 25 μg ^{15}N. ml^{-1} nitrate solutions respectively (calculated from the rate of water uptake by detached leaves of 0,39 ml. $gFW^{-1}.hr^{-1}$). Considering the short-term nature of the experiments and the consequent dilution of the ^{15}N-nitrate, one would not expect to assess absolute assimilation rates using this method.

3.3.2 Vacuum Infiltration

Whole leaves of Datura were vacuum infiltrated with solutions of 400 μg ^{15}N. ml^{-1} potassium nitrate. 5 mM MSO was included in the infiltration medium of those leaves that had received a 2 hr pre-treatment with the inhibitor. Leaves were completely immersed in the infiltration medium and degassed under vacuum ($\pm$2 kPa) for 3 mins. Having ensured that the leaves were completely submerged, release of the vacuum resulted in the almost immediate, visual penetration of the feeding solution into the intercellular spaces. Thus the disadvantageous time lag of transpirational feeding is avoided and the use of 400 μg ^{15}N. ml^{-1} nitrate minimises the dilution effect. The essentially anaerobic conditions produced at the time of infiltration do not appear to impair photosynthesis in short-term experiments (C. F. Cresswell, pers. comm.).

Once infiltrated, the leaves were returned to the growth cabinet and transpirationally fed with either a 200 μg. ml^{-1} or a 25 μg. ml^{-1} nitrate-^{15}N solution, depending on the pre-treatment. Leaf tissue was not left to photosynthesize for longer than 60 mins because it was considered that the elevated intracellular nitrate concentration

would, with time, alter the relevant enzyme activities to levels uncharacteristic of the pre-treatment conditions.

3.3.3 Pressure Infiltration

A method employing the Scholander bomb to effectively force the feeding solution into root tissues was developed in response to the inefficiency of vacuum infiltration techniques with whole Datura roots. Feeding the isotope hydroponically, by substitution of the ^{14}N-nitrate with ^{15}N-nitrate in the nutrient solution, also proved unsatisfactory, yielding non-reproducible results.

Roots were placed in a full nutrient solution containing 400 $\mu g.\ ml^{-1}$ nitrate ^{15}N (with and without MSO) in a beaker inside the bomb with the severed end of the stem projecting out the lid. The system was then subjected to a pressure of 1000 kPa for 5 mins during which time $\pm 0{,}5$ ml of sap exuded from the cut surface. On release of the pressure and removal from the bomb the root material was transferred to a 100 $\mu g\ ^{15}N.\ ml^{-1}$ nitrate nutrient solution (with and without 5 mM MSO) for 5, 15 and 30 mins.

3.4 Extraction

The fresh weight of transpirationally fed leaves was determined immediately on harvesting. Infiltrated material was weighed prior to isotope application and the weights corrected for discarded tissue after infiltration. Transpirationally fed leaves were killed by homogenizing in cold 80% ethanol (1 g tissue. 50 ml $ethanol^{-1}$) with

an Ultra-Turrax homogenizer. Infiltrated material was killed in liquid nitrogen prior to homogenization to maintain the strict time course nature of such experiments. After homogenization, leaf and root tissue was extracted in 80% ethanol at 0°C for 24 hrs. The homogenate was filtered through Whatman No. 1 filter paper and the alcoholic extract evaporated under an airstream to a final volume of 10 ml. Chlorophyll and lipid material were removed by shaking the extract with ±5 ml of petroleum ether. Samples were then frozen and the petroleum ether poured off.

3.5 Quantitative Determination and Separation of the Soluble Amino Compounds

A Beckman 120C automatic amino acid analyser was used for ion exchange chromatography of the plant extracts. Acidic and neutral amino acids were separated and their concentrations determined on a 69 cm column of Beckman PA35 spherical ion-exchange resin, employing, sequentially, two lithium citrate buffers (0,03 N Lithium) of pH 2,72 (0,05 M citrate) and pH 3,73 (0,21 M citrate), respectively (Atkin and Ferdinand, 1970; Kedenberg, 1971). Lithium buffers were used for quantitative and preparative runs to facilitate separation of the amides, glutamine and asparagine. Basic amino acids were chromatographed on a short 12 cm column (PA35 resin) employing 0,35 N sodium buffer at pH 5,83 (Atkin and Ferdinand, 1970; Kedenberg, 1971).

The separated components were automatically subjected to reaction with ninhydrin at 100°C and the optical densities of the amino acid-ninhydrin reaction products were recorded on a Honeywell Electronik 16 logarithmic recorder and a Beckman 125 digital integrator. The

quantitative value for each amino acid was calculated from the optical density value, obtained from the digital integrator, and a specific conversion constant, determined for each amino acid from calibration runs.

Isolation and collection of acidic and neutral amino compounds, and ammonia, were performed on a 150 cm preparative column (M84 ion exchange resin) and a 12 cm column (PA 35 resin, respectively. A stream divider system was used in conjunction with preparative runs to split the eluate stream in the ratio 1 (analytical system) : 10 (collection system). The eluate was collected automatically in test tubes by an LKB Ultrorac 7000 fraction collector. The amino compound pools were identified by spotting 0,2 ml of the eluate from each test tube on 32 cm Whatman No. 41 filter paper and spraying with chromatographic ninhydrin spray. After allowing the colour of the amino acid-ninhydrin reaction product to develop (at 110°C for 15 mins) one could visually compare the colour intensities obtained with the print-out from the recorder and locate the amino compounds.

3.6 ^{15}N Analysis

3.6.1 Sample Preparation

The separated amino compounds were reduced in volume to 10 ml and converted to ammonium sulphate by Kjeldahl digestion using BDH mercury catalyst tablets. One tablet, containing the equivalent of 0,1 g mercury and 1,0 g sodium sulphate and 3 ml of concentrated sulphuric acid (N free), was added to each Kjeldahl flask. The sodium sulphate served to speed up the digestion process by elevating the temperature (Kirk, 1950; McKenzie and Wallace, 1954).

Ammonia was distilled off in a Markam micro-Kjeldahl still after alkalising with 15 ml of 50% sodium hydroxide. The possible loss of ammonia, through its binding with the mercuric oxide precipitated on addition of the alkali (McKenzie and Wallace, 1954), was not a serious drawback in the present experiments where the quantitative recovery of ammonia during distillation was not essential. The ammonia distillate was collected in 2 ml of 0,02 N hydrochloric acid and the amount of ammonia determined by titration with standard 0,005 N sodium hydroxide using Tshiro's indicator. With the ^{15}N analytical system used in the present experiments, a minimum of 15 µg ammonium-N was required in each sample. In most cases, however, amounts in excess of the optimum 30 µg ammonium-N, were obtained.

3.6.2 ^{15}N Determinations

After titration, the sample was acidified with 0,5 ml of 0,1 N hydrochloric acid to prevent the loss of ammonia. An over-acidified sample on reaction with sodium hypobromite in the final oxidation step caused copious quantities of bromine to evolve, leading to grossly erroneous ^{15}N determinations. The acidified sample was reduced in volume in preparation for ^{15}N analysis by atomic emission spectroscopy.

The sample chemistry followed was based on that described by Faust (1967) with the use of an alkaline hypobromite solution as the oxidant. The hypobromite reacted with the sample under vacuum to release nitrogen gas according to the reaction :

$$2NH_3 + 3NaOBr \longrightarrow N_2 + 3H_2O + 3NaBr \quad \text{(Kamen, 1957)}$$

The vacuum system comprised an Edwards high vacuum pump to establish a

good pre-vacuum (0,1 kPa) connected in series with a mercury diffusion pump (Packard), to bring the system to a final pressure of 1,0 Pa. The vapour pressure of the system was reduced by two liquid nitrogen cold traps.

3.6.3 Calculation and Expression of ^{15}N Results

The discharge tube containing the sample as N_2 was 'fired' in a Statron (Packard) NOI-4 atomic emission spectrophotometer (A.E.S.). A typically red/violet colour was obtained on firing. A blue colour indicated the presence of interfering water vapour or bromine. This method is based on the photoelectric recording of the bandheads emitted by the three isotopic molecules $^{15}N^{15}N$, $^{14}N^{15}N$ and $^{14}N^{14}N$ on firing. A typical trace showing good separation of all three hybrid peaks is shown in Fig. 1. Enrichments were calculated from the formula :

$$En\% = \frac{100}{2(A/B + Vb/Va)} + 1$$

where A and B are the bandheads of the $^{14}N^{14}N$ and $^{15}N^{14}N$ molecules (Fig. 1) respectively, and Va and Vb are the gain settings on the A.E.S. at which the bandheads A and B are recorded.

All ^{15}N enrichments obtained in this manner were corrected from a calibration curve drawn up periodically for the Statron. The percentage enrichment in excess of the natural abundance (A%E) was obtained by subtracting the natural abundance (0,37%) from the corrected percentage enrichment (Yoneyama et al., 1975). The pool sizes of the individual amino compounds were expressed in micromoles per gram fresh weight (μmole. gFW^{-1}) and were determined by ion exchange chromatography

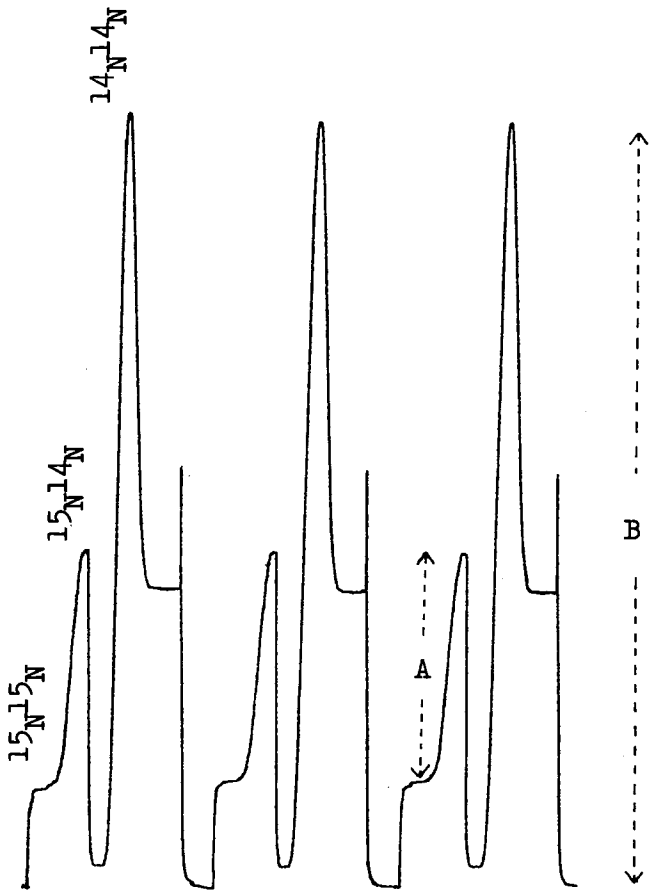

FIG. 1 : Typical traces showing good separation of the three hybrid molecules of nitrogen : $^{15}N^{15}N$, $^{15}N^{14}N$, $^{14}N^{14}N$. A and B represent the peak heights of the $^{15}N^{14}N$ and $^{14}N^{14}N$ bandheads, respectively.

as described previously (section 2.5). The ^{15}N content of a particular amino pool, expressed as micrograms ^{15}N per gram fresh weight ($\mu g\ ^{15}N.\ gFW^{-1}$), was calculated by multiplying the pool size by the A%E value and the number of nitrogen atoms per molecule of the respective amino compound. This latter figure proved a more useful expression of the ^{15}N results than the A%E value, in the present study.

3.7 Hydrolysis of Glutamine

In order to estimate the relative enrichment of the amido and amino nitrogen of glutamine, half of the amide, after separation on the amino acid analyser, was transferred to a hydrolysis flask. Approximately 20 ml of 6 N hydrochloric acid was added to the contents of the flask. The air inside the hydrolysis flask was displaced by nitrogen before it was sealed. The sealed flask was then placed in an oven at 110°C for 24 hrs. Under these conditions the amide hydrolyses to form ammonia and glutamate.

After cooling and ammonia distillation (section 3.6.1) the glutamate-fraction was removed from the still and neutralized by slow addition of concentrated hydrochloric acid. This fraction was then reduced in volume and subjected to Kjeldahl digestion and ammonia distillation. The ammonia and glutamate fractions were analysed as amido and amino nitrogen respectively.

3.8 Enzyme Assays

Assays for nitrate reductase and glutamine synthetase were performed on Datura leaves. Glutamate dehydrogenase activity was

determined in both the roots and the leaves. Attempts to detect a NAD(P)H-dependent glutamate synthase in leaf tissue, using the reaction mixture of Dougall (1974), proved unsuccessful.

3.8.1 Extraction Procedures

3.8.1.1 Leaf Tissue

An imidazole extraction buffer (Rhodes et al., 1975) comprising 0,05 M imidazole-hydrochloric acid (pH 7,2), 0,5 mM ethylenediamine tetra-acetic acid and 1,0 mM dithiothreitol, proved to be suitable for all leaf enzyme assays.

One gram of leaf material was ground up in a chilled (0-4°C) mortar and pestle. The homogenate was then poured through two layers of cheesecloth and centrifuged at 1000 X g for 5 mins in a Beckman J-21 refrigerated centrifuge. The supernatant was used as the crude enzyme source for both glutamine synthetase and nitrate reductase assays. In the glutamate dehydrogenase assay 0,01 ml of Triton X-50 was added to the supernatant after the initial centrifugation, and the solution was allowed to stand for 10 mins. Final clarification of this extract was carried out at 29000 X g for 15 mins and the supernatant was assayed for glutamate dehydrogenase activity (Joy, 1969).

Triton, a non-ionic detergent of the polyethylene glycol type, has been used extensively in the selective solubilization of biological materials (eg. Vernon et al., 1966). It is thought to cause disintegration of membranes by removing the lipid component, solubilization, however, being incomplete (Bottomley, 1970). Treatment with Triton X-50 was essential for the detection of glutamate

dehydrogenase activity in the current experiments.

3.8.1.2 Root Tissue

A crude total extract of Datura roots was prepared according to the method of Pahlich and Joy (1971). Root tissue was washed in distilled water and after being blotted dry was ground in a chilled mortar with 0,05 M tris, pH 7,5; containing 0,4 M sucrose. The ratio of root tissue to extraction buffer was 1 g : 2,5 ml. After filtering through cheesecloth the homogenate was centrifuged for 12 mins at 1000 X g. The sediment was washed three times in grinding medium (total volume of 10 ml) and the washings were combined with the supernatant fraction. Triton X-50 was added to make a final solution of 0,1% and the extract was shaken and put aside on ice for 30 mins. This solution was then clarified at 25000 X g for 10 mins. All enzyme preparations, buffers and glassware were maintained at 0-4°C throughout extraction.

3.8.2 Reaction Media

3.8.2.1 Nitrate Reductase

A time course study was performed on the induction of nitrate reductase activity at the 25 μg. ml^{-1} and 200 μg. ml^{-1} nitrate-N feeding levels. The assay mixture used was based on that described by Bar Akiva and Sagiv (1967) and comprised : 0,1 ml 1,0 M phosphate buffer, pH 7,4; 0,4 ml 2,82 mM NADH; 0,2 ml 0,1 M potassium nitrate; 1,1 ml of distilled water and 0,2 ml of enzyme extract. The reaction was started by the addition of the enzyme extract and was allowed to

proceed for 30 mins at 30°C in a shaking water bath. The optical density was read at 540 nm in a Bausch and Lomb Spectronic 20 spectrophotometer after the addition of 1 ml of 1,0% (w/v) sulphanilamide-hydrochloric acid (1 : 4) and 1,0 ml of 0,01% N (1-naphthyl) ethylenediamine dihydrochloride. The quantity of nitrate produced was calculated after the comparison of the optical density readings obtained experimentally with standard curves obtained for complete reaction mixtures containing known amounts of potassium nitrite.

3.8.2.2 Glutamine Synthetase

A similar time course experiment was performed on glutamine synthetase activities at the two feeding levels. The reaction mixture (pH 7,2) contained 36 µmole adenosine triphosphate, 90 µmole magnesium sulphate, 12 µmole hydroxylamine, 184 µmole L-glutamate and 100 µmole imidazole-hydrochloric acid, in a final volume of 2 ml (Rhodes et al., 1975). The reaction was initiated by the addition of 0,2 ml of enzyme extract and was incubated for 30 mins in a shaking water bath at 30°C. The reaction was terminated by the addition of 1 ml of a mixture of trichloroacetic acid and 8 g of ferric chloride in 250 ml 0,5 N hydrochloric acid (Ferguson and Sims, 1971). The optical density was read at 500 nm in a Spectronic 20 spectrophotometer and glutamine synthetase activity was estimated from the amount of hydroxymate formed according to the reaction :

$$\text{L-glutamine + hydroxylamine + ATP} \xrightarrow[\text{synthetase}]{\text{glutamine}} \text{glutamylhydroxymate + } NH_4^+$$

(Hubbard and Stadtman, 1967). The optical densities obtained experimentally were compared with those measured for the complete reaction mixture

containing known amounts of L-glutamate and monohydroxymate.

3.8.2.3 Glutamate Dehydrogenase

The effect of MSO on glutamate dehydrogenase activity was investigated in both the leaves and the roots. The pre-treatment period was similar to that described for the ^{15}N feeding experiments (section 2.2). The reductive amination assays of Joy (1969) and Pahlich and Joy (1973) were used for the leaves and roots respectively.

The assay medium for the leaf tissue contained : 1,9 ml of buffer; 0,2 ml 1,0 M ammonium sulphate; 0,2 ml 0,2 M 2-oxoglutarate adjusted to pH 7,5 with phosphate buffer; and 0,2 ml of enzyme extract. For root GDH assays the reaction mixture comprised : 2,0 ml 0,2 M buffer; 0,2 ml 0,2 M oxoglutarate (pH 7,5); 0,2 ml 3,2 M ammonium sulphate; and 0,1 ml of extract. Assay buffers used in both leaf and root GDH assays were : 0,2 M tris (pH 8,4) for the NADH-specific activities and 0,1 M phosphate (pH 7,5) for NADPH-dependent activities. The assay mixture plus extract was incubated for 2 mins at 30°C prior to spectrophotometric measurements.

The reaction was initiated by the addition of 0,5 ml of 1,0 mM NAD(P)H and the decrease in absorbance at 340 nm was monitored on a Unicam SP 1800 ultraviolet spectrophotometer. A Unicam linear recorder connected to the spectrophotometer enabled continuous recording of the rate of oxidation of the pyridine nucleotides. Blanks lacking either 2-oxoglutarate or ammonium were run for each assay. Optical density readings measured in the assays were compared with those obtained for known quantities of exogenous NAD(P)H in the reaction mixture.

3.9 Xylem Sap Analysis

3.9.1 Collection of Bleeding Sap

Seedlings were grown hydroponically for 4-5 weeks at 50, 100 and 200 μg N. ml^{-1} nitrate (Tables 1 and 2). Plants were then detopped approximately 2 cm above the root system and the sap, exuded through the action of root pressure, was collected in a collar of Tygon tubing fitted over the cut stem. The liquid exuding from the cut surface is regarded as originating from xylem vessels (Pate, 1962) which are believed to provide the main passage for the upward movement of dissolved nitrogenous compounds within the plant (Pate, 1973). All sap collections were performed during midsummer and at midday to minimize the effect of seasonal and diurnal variations in the amino compounds being transported (Pate, 1962). Only the sap exuded during the first two hours after detopping was collected because of starvation effects which become apparent within a few hours of removal of the shoot (Koster, 1963; Pate and Wallace, 1964).

Sap samples were stored frozen and later analysed for the acidic, neutral and basic amino compound fractions on a Beckman 120 C amino acid analyser (Section 2.5). Nitrate determinations were also performed on sap samples as described in the following section.

3.9.2 Determination of Nitrate

The concentration of nitrate in the bleeding sap was determined with an Orion liquid ion exchange nitrate electrode. Sap samples were stored frozen prior to measurements. A calibration curve was constructed

on each day of measurement using sodium nitrate standard solutions. Standardizing solutions were treated with 10 µg. ml^{-1} phenyl mercuric acetate preservative to inhibit biological growth. Seeing that the response of the electrode to nitrate was logarithmic and not strictly linear at high concentrations, samples were diluted with distilled water to lower the nitrate levels into the more sensitive and linear response region of the electrode (10 - 50 µg N. ml^{-1} nitrate).

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Xylem Sap Analysis

As a preliminary study, analyses of the bleeding sap of 4-5 week old Datura plants grown at three different nitrate concentrations; 50, 100 and 200 µg. ml^{-1} nitrate N, were performed. The collection and analysis of the bleeding sap was as described in section 3.9. In addition, a leaf sample was taken for each corresponding sap collection and was subjected to automatic amino acid analysis (section 3.5). Two different plants were analysed from each nitrate feeding level to duplicate results. The pool sizes for sap and leaf results are represented in Table 3. It was assumed that the distribution of solutes present in the bleeding sap gave a true picture of what normally ascends in the xylem. This method of sap collection, though subject to certain criticisms and inherent disadvantages, can yield much information concerning the assimilatory capacity of roots and the distribution of nitrogen and carbon amongst the translocated solutes reaching the leaves.

Certain plants possess the ability to accumulate nitrate from the external medium against the concentration gradient and apparently an electrochemical gradient (Bowling et al., 1966). This phenomenon was most marked in Datura at the low feeding level where the ratio (NO_3^-) xylem sap/(NO_3^-) in the nutrient solution is between 5,8 and 6,0. At the 200 µg N. ml^{-1} feeding level this ratio drops to between 2,0 and 2,5. Ivanko and Ingversen (1971) have reported similar ratios between 1,2 and 1,4 for maize while Ricinus communis shows massive accumulation

TABLE 3 : The levels of amino compounds, nitrate and ammonia present in the xylem sap and leaves of Datura stramonium grown in liquid culture at three nitrate regimes.

	50 µg N. ml^{-1} Feeding Level				100 µg N. ml^{-1} Feeding Level				200 µg N. ml^{-1} Feeding Level			
	Plant I		Plant II		Plant I		Plant II		Plant I		Plant II	
	Sap µgN.ml^{-1}	Leaf µgN.gFW^{-1}	Sap µgN.ml^{-1}	Leaf µgN.gFW^{-1}	Sap µgN.ml^{-1}	Leaf µgN.gFW^{-1}	Sap µgN.ml^{-1}	Leaf µgN.gFW^{-1}	Sap µgN.ml^{-1}	Leaf µgN.gFW^{-1}	Sap µgN.ml^{-1}	Leaf µgN.gFW^{-1}
NO_3	290,00		300,00		285,00		375,00		400,00		490,00	
NH_4	13,24	8,78	6,90	9,99	14,46	6,66	16,48	5,40	29,34	16,87	36,46	11,19
ASP	0,27	14,06	0,18	16,04	0,60	20,22	1,78	10,07	0,48	37,40	0,50	24,64
THR	1,99	6,31	1,44	5,46	2,14	3,58	2,42	2,30	1,41	16,24	0,92	6,78
SER	0,67	6,48	0,59	5,29	0,27	3,92	0,63	2,44	0,66	13,16	0,10	4,93
ASG	2,02	1,13	0,91	t	2,30	3,28	2,38	1,17	1,16	19,31	1,15	1,67
GLU	0,29	30,17	0,21	39,63	0,13	30,24	0,87	13,68	1,10	51,21	0,18	30,83
GLN	13,16	17,01	12,21	9,52	10,19	5,07	9,63	3,08	2,16	40,68	14,70	6,88
GLY	0,70	2,62	0,03	0,48	-	0,15	0,07	0,05	-	1,11	-	0,26
ALA	0,76	10,70	0,43	7,95	0,042	4,69	0,24	1,56	0,11	6,76	-	6,45
VAL	1,54	-	1,27	-	-	-	-	-	-	0,13	-	-
MET	0,42	-	0,42	-	-	-	-	-	-	-	-	-
ILE	1,09	0,45	0,84	0,48	2,23	0,30	1,74	0,27	1,23	3,74	0,56	0,26
LEU	0,81	0,29	0,59	0,15	1,22	0,21	0,90	0,10	0,80	1,00	0,38	0,45
TYR	0,24	-	0,11	-	0,14	-	0,10	-	-	1,76	0,03	-
PHE	0,14	0,98	0,14	1,01	0,17	-	0,15	-	-	0,53	0,04	0,17
LYS	6,27	1,08	5,04	0,69	12,96	0,93	10,28	0,20	8,06	3,74	5,10	2,05
HIST	5,29	0,29	4,12	-	8,19	0,22	5,92	0,07	4,12	1,89	3,70	0,14
ARG	9,80	0,20	8,06	-	7,45	1,12	5,32	0	3,75	0,58	3,25	0,67

t = trace

of nitrate, the concentration in the sap being some 10 times that in the external solution (Bowling et al., 1966).

Nitrate forms the major single constituent of the xylem sap of Datura, representing some 82%-88% of the total nitrogen moving up in the xylem stream. This finding contrasts with that of Bollard (1957) who found the xylem sap in nearly all plants investigated contained mainly organic nitrogen. It is now a well-established fact that plants exhibit a wide spectrum of nitrate concentrations in their xylem stream (Wallace and Pate, 1967) ranging from Xanthium on the one extreme, having some 98% of its sap nitrogen as free nitrate (Wallace and Pate, 1967), to Lupinus on the other extreme having only 7% nitrogen as nitrate (Pate, 1973). Species such as Lupinus with low nitrate levels in the xylem sap are characterized by a very active nitrate reducing and assimilation system in the roots and consequently high levels of organic nitrogen in their xylem. Datura appears to lie near the other end of the spectrum where nitrate is reduced and assimilated predominantly in the leaf. Nevertheless, the results (Table 3) suggest Datura roots are capable of inorganic nitrogen assimilation which, in the present experiments, appears to be in excess of its current demands for growth.

Weissman (1964) and Pate (1968) have shown the nitrate content of xylem sap to be subject to considerable changes in response to variations in the concentration of nitrate in the feeding solution. Increasing the external concentration of nitrate in the present experiments causes a substantial increase in the amount of nitrate in the xylem stream (Table 3), though on a total N percentage basis the nitrate concentration remains within relatively small limits

(82%-88%). At the higher feeding level one also finds the relative concentration of ammonia increased slightly while the percentage nitrogen tied up in organic union as amino compounds decreased sharply. These findings suggest that there is an increase in the activity of the nitrate-reducing enzymes in the root with an increase in the nitrate feeding level. The enzymes of ammonia assimilation, however, do not appear to parallel the increase in activity of nitrate and nitrite reductase and there is a decrease in the export of amino nitrogen from the root to the leaf at the high feeding level. This decrease in the amino nitrogen levels and increase in nitrate and ammonia concentrations in the xylem sap from plants fed 200 μg N. ml^{-1} suggest an even greater role for the leaf in nitrogen assimilation compared to the lower feeding level. A similar situation exists in Pisum arvense where a supply of nitrate in excess of the assimilatory capacities of the root serves to reduce the dependence of the shoot on the root for organic nitrogen (Wallace and Pate, 1967).

The most common translocated amino compounds in Datura xylem sap include those amino compounds with a carbon/nitrogen ratio of three or less and they account for approximately 80% of the amino nitrogen. It seems logical that the root, which relies heavily on the shoot for the supply of carbon skeletons for nitrogen assimilation (Dixon, 1976), must minimize the loss of carbon to the shoot. Translocation of solutes with a low carbon/nitrogen ratio provides the root with just such a mechanism. It is interesting to note that whereas labelled sugars received by the root are utilized as a carbon source for the synthesis of amino compounds, newly absorbed as opposed to translocated nitrogen is used in the process (Pate, 1973).

Of the major xylem stream translocated amino compounds in Datura, including the basic amino acids lysine, histidine and arginine and the amide glutamine, only the latter is present in comparable amounts in the free amino compounds of the leaf. Asparagine, a common amide of nitrogen transport of legumes, eg. Pisum arvense (Pate and Wallace, 1964), trees, eg. apple (Tromp and Ovaa, 1976), and in fact, most plants (Lea and Fowden, 1975a) is present only in very small amounts in the xylem sap of Datura, representing some 0,03%-0,66% of the total nitrogen. In Datura glutamine constitutes the major organic constituent of the xylem stream at both the high and low feeding level, similar to findings in a number of varieties of sugar cane (Waldron, 1976). At the intermediate, 100 μg N. ml^{-1}, feeding level, however, a slightly higher percentage of the organic nitrogen is translocated as lysine.

The substantial decrease in the relative amounts of the basic amino acids in the leaf below that in the sap indicates either a channelling towards leaf protein synthesis or active metabolism of these compounds in the leaf. There is substantial synthesis of the acidic amino acids, glutamate and aspartate in the leaf at all nitrate feeding levels. Both were present in only very small amounts in the sap. Other important free leaf amino acids which showed increased relative amounts in the leaf over that in the sap included alanine, serine and threonine. A similar situation exists in the tomato (Lorenz, 1976), which belongs to the same family as Datura, and in Xanthium (Wallace and Pate, 1967); both of which exhibit an extensive synthesis of aspartate, glutamate, alanine, serine and threonine in the shoot system.

4.2 The Synthesis of the Major Soluble Leaf Amino Compounds

4.2.1 The Synthesis of Glutamine and Glutamate

The routing of ^{15}N to glutamine and glutamate was monitored following the feeding of ^{15}N-labelled nitrate and glutamine (amide ^{15}N) to the leaf, either in the xylem stream or by vacuum infiltration. The relative ^{15}N content of these amino compounds over a range of feeding times was assumed to be indicative of the relative *in vivo* activity of the two major pathways of ammonia assimilation (Chapter 2). Further investigations of the participation of GS in ammonia assimilation were provided by ^{15}N feeding experiments utilizing methionine sulphoximine (MSO) as a GS inhibitor.

4.2.1.1 Nitrate ^{15}N Feeding Experiments

4.2.1.1.1 Xylem Stream Feeding Experiments

Leaves of *Datura* were fed through the petiole with 200 µg. ml^{-1} and 25 µg. ml^{-1} nitrate-^{15}N solutions after a 4 hr induction period at the same respective concentrations of ^{14}N nitrate (see 3.3.1). The percentage enrichments and ^{15}N content of glutamine and glutamate for these 7, 17 and 45 min feeding experiments are shown in Table 4. It is interesting to note that the incorporation of newly reduced nitrogen into glutamate and glutamine appears to be markedly affected by the feeding level. A consideration of the ^{15}N content of the two pools reveals a greater accumulation of ^{15}N in glutamine than in glutamate at the higher feeding level. The ^{15}N content of glutamate only approaches that of glutamine after 45 mins at this feeding level.

On the other hand, at the lower, 25 μg N. ml^{-1} nitrate feeding level, the reverse is true; the routing of ^{15}N to glutamate being some two times higher than to glutamine throughout the time course of the experiment. These findings suggest that at the higher feeding level the GS/GOGAT pathway predominates, resulting in the high initial rate of ^{15}N accumulation in glutamine. At the lower feeding level, it is possible that GDH has assumed the major ammonia assimilatory role resulting in the higher initial routing of ^{15}N into glutamate. Such an interpretation is in direct contrast with the view that GS, rather than GDH, by virtue of its higher affinity for ammonia, is better suited to assimilation under conditions of restricted ammonia availability (2.5). Some fungi, however, do exhibit a similar response to nitrogen feeding concentration as is apparently shown by Datura. Growth of Aspergillus nidulans and Neurospora crassa on high concentrations of ammonia resulted in low mycelial concentrations of GDH but high concentrations of GS (Pateman, 1969). This finding is in agreement with the marked depression of the NADP-linked GDH of ammonia limited N. crassa cultures found by Barrat (1963). Similarly, in a number of yeasts the highest cellular content of biosynthetic, NADP-dependent GDH was found in nitrogen limited chemostat cultures (Brown and Johnson, 1970; Burn et al., 1974). It appears that in the fungi, a group of organisms where ammonia assimilation depends on GDH (Meers et al., 1974) the synthesis of the principal ammonia assimilatory enzyme is highest under conditions of nitrogen limitation, thereby compensating for its relatively poor substrate affinity (Burn et al., 1974). Such a system is acceptable for an organism in which GDH provides the sole route for

TABLE 4

^{15}N enrichments and distribution in the soluble glutamine and glutamate pools of Datura stramonium leaves fed 200 µg $^{15}N.ml^{-1}$ and 25 µg $^{15}N.ml^{-1}$ nitrate through the xylem stream for 7, 17 and 45 min. The leaves were treated with 200 µg $N.ml^{-1}$ and 25 µg $N.ml^{-1}$ nitrate for 5 hr, prior to ^{15}N feeding.

200 µg ^{15}N ml^{-1} Feeding Level

	7 min		17 min		45 min	
	GLN	GLU	GLN	GLU	GLN	GLU
Enrichment (A%E)	0,48	0,72	2,14	1,41	4,33	12,21
Concentration in the leaf (µm.gFW^{-1})	5,601	3,835	5,949	4,108	6,131	3,154
^{15}N Content (µg $^{15}N.gFW^{-1}$)	0,754	0,437	3,556	0,815	6,666	5,278

25 µg $^{15}N.$ gFW^{-1} Feeding Level

	7 min		17 min		45 min	
	GLN	GLU	GLN	GLU	GLN	GLU
Enrichment (A%E)	0,20	0,63	0,33	0,68	0,76	2,55
Concentration in the leaf (µm.gFW^{-1})	1,948	2,487	1,424	5,209	1,913	2,529
^{15}N Content (µg $^{15}N.gFW^{-1}$)	0,116	0,235	0,132	0,492	0,402	0,921

2-amino production from ammonia (GOGAT has not as yet been demonstrated in a fungus). There is, however, no logical reason why higher plants should adopt a similar strategy when they possess an alternative enzyme system capable of scavenging low concentrations of ammonia.

Another interpretation of the findings reported in Table 3 does, however, exist. It is possible that there is a small, actively turning-over pool of glutamine in the chloroplast (Bassham and Kirk, 1964) which saturates quickly with ^{15}N from nitrate-^{15}N reduction and the activity of which is masked by a large storage pool of ^{14}N-glutamine outside the chloroplast.

Thus, the effect of feeding level on the relative rates of ^{15}N incorporation into glutamine and glutamate can be explained either by a shift from one pathway of ammonia assimilation to another, or by the masking effect of ^{15}N incorporation into glutamine by the presence of a large glutamine pool synthesized during the pretreatment period at the high feeding level.

To test these hypotheses the previous experiment was repeated using infiltration technique (to avoid the undesirable time lag of transpirational feeding techniques) and the results compared with those obtained from experiments in which GS activity was inhibited by MSO. These experiments are reported in sections 4.2.1.1.2 and 4.2.1.1.3.

4.2.1.1.2 Vacuum Infiltration Experiments Using Nitrate-^{15}N

Leaves of Datura were vacuum infiltrated with 400 μg $^{15}N.ml^{-1}$ nitrate after a 6 hr induction period at either 200 $\mu g.\ ml^{-1}$ or 25 $\mu g.\ ml^{-1}$ nitrate nitrogen (described in 2.3.2). After this period

of infiltration the leaves were allowed to photosynthesise in a controlled environment chamber for 5, 15 and 30 min under the conditions already described in 3.2. prior to harvesting and extraction. Subsequent ^{15}N analysis of the separated commonest free amino acid and ammonia pools is summarised in Table 5.

A similar trend to that found for transpirationally fed leaves is revealed. Leaves given a high nitrate treatment show a major routing of ^{15}N to glutamine (Fig. 2a) while low nitrate induced leaves synthesise predominantly glutamate from newly reduced nitrogen (Fig. 2b). Hydrolysis of glutamine from the 30 min ^{15}N-feeding experiment at both feeding levels shows the amide nitrogen to be more enriched with ^{15}N than the amino nitrogen (Table 6), a finding consistent with the synthesis of this amide via GS. It is interesting to note that while the ^{15}N content of glutamate and glutamine shows a differing response to feeding level, the actual enrichment (A%E) of glutamate is consistently higher than that of glutamine at both feeding levels. Glutamate has similarly been found to have a consistently higher percentage enrichment in ammonia-^{15}N fed Chlorella (Bassham and Kirk, 1964) and nitrate-^{15}N fed sunflower leaves (Ito and Kumazawa, 1976).

The ammonia pools at both feeding levels appear to saturate rapidly after 15 min at a low ^{15}N enrichment value, indicating the existence of more than one ammonia pool. It is probable that there is a small actively turning-over pool in the chloroplast produced by nitrate reduction, and a large pool derived from amino acid metabolism in the cytosol. The size limits of the smaller assimilatory pool are most likely determined by the rate of nitrate reduction and ammonia

TABLE 5 : ^{15}N enrichments and distribution in the commonest free amino compounds of Datura stramonium leaves after feeding KNO_3 at 25 and 200 µg N.ml^{-1} levels for 8 hr followed by $K^{15}NO_3$ infiltration and subsequent photosynthesis for 5, 15 and 30 min periods.

200 µg N ml^{-1} Feeding Level

	5 min			15 min			30 min		
	Enrich-ment (A%E)	Concentration in leaf (µm.gFW^{-1})	^{15}N content (µg^{15}N.gFW^{-1})	Enrich-ment (A%E)	Concentration in leaf (µm.gFW^{-1})	^{15}N content (µg^{15}N.gFW^{-1})	Enrich-ment (A%E)	Concentration in leaf (µm.gFW^{-1})	^{15}N content (µg^{15}N.gFW^{-1})
ASP	0,57	1,215	0,097	2,15	0,950	0,286	4,51	1,396	0,880
ASN	0,19	0,429	0,022	0,45	0,445	0,056	0,94	0,730	0,192
ALA	0,44	0,614	0,037	0,71	0,388	0,039	1,85	0,528	0,137
SER	0,58	2,300	0,187	3,05	2,622	1,120	4,20	2,632	1,548
THR	0,23	0,335	0,011	0,32	0,401	0,018	-	0,346	-
GLY	0,45	1,644	0,104	1,98	2,452	0,680	2,25	1,817	0,573
GLN	0,63	7,162	1,263	1,05	7,783	2,288	2,10	8,910	5,239
GLU	1,80	3,906	0,984	3,17	3,883	1,723	6,73	3,640	3,430
NH_3	1,22	1,251	0,214	2,39	1,789	0,599	1,62	1,728	0,392

25 µg N ml^{-1} Feeding Level

	5 min			15 min			30 min		
	Enrich-ment (A%E)	Concentration in leaf (µm.gFW^{-1})	^{15}N content (µg^{15}N.gFW^{-1})	Enrich-ment (A%E)	Concentration in leaf (µm.gFW-1)	^{15}N content (µg^{15}N.gFW^{-1})	Enrich-ment (A%E)	Concentration in leaf (µm.gFW^{-1})	^{15}N content (µg^{15}N.gFW^{-1})
ASP	0,30	0,413	0,017	2,22	0,393	0,122	7,13	1,300	1,298
ASN	0,15	0,146	0,006	0,44	0,057	0,007	1,41	0,366	0,145
ALA	0,73	0,333	0,034	-	0,087	-	3,05	0,309	0,132
SER	0,94	1,132	0,149	4,70	1,057	0,696	6,23	1,780	1,553
THR	0,26	0,406	0,015	0,53	0,423	0,031	-	0,421	-
GLY	0,82	1,142	0,131	3,19	1,057	0,472	5,08	1,097	0,780
GLN	0,55	3,585	0,552	1,19	3,185	1,061	3,34	3,719	3,478
GLU	2,74	2,628	1,008	6,95	2,611	2,540	10,93	2,941	4,500
NH_3	1,53	1,206	0,258	2,65	2,215	0,822	4,27	1,395	0,834

TABLE 6

The percentage enrichments (A%E) for the amido and amino nitrogen of glutamine separated by acid hydrolysis.

	Feeding Level	
	200 µg N. ml^{-1}	25 µg N. ml^{-1}
amide N	2,71	2,13
amino N	1,19	0,72

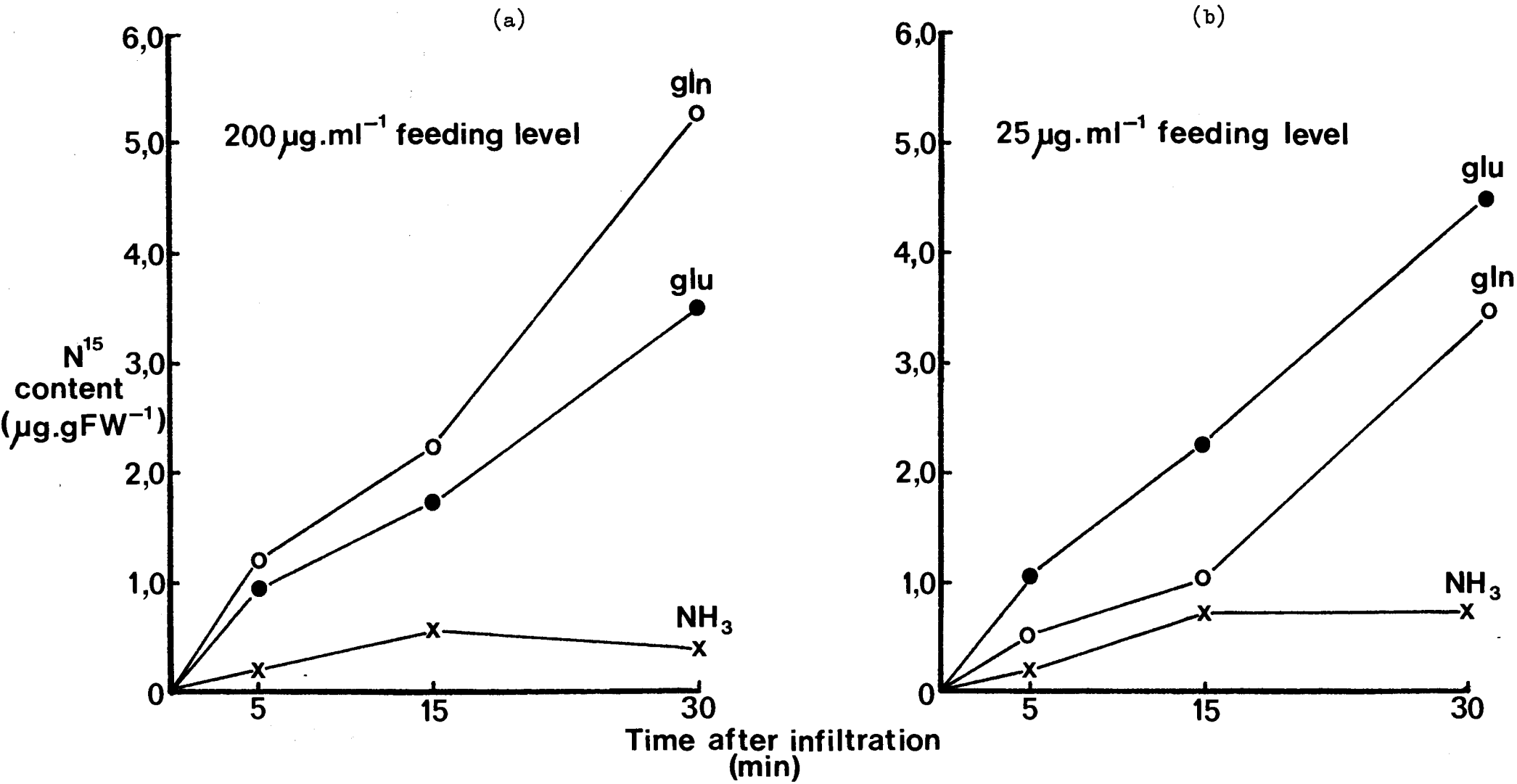

FIG. 2 : The time course of ^{15}N incorporation into the soluble glutamine (O——O), glutamate (●——●) and ammonia (X——X) pools of Datura leaves. The leaves were fed via their xylem streams with either 200 µg N. ml^{-1} or 25 µg. ml^{-1} potassium nitrate for 6 hr prior to infiltration. Leaves were infiltrated with 400 µg ^{15}N. ml^{-1} nitrate and allowed to photosynthesise for 5, 15 and 30 min.

assimilation into amino acids.

4.2.1.1.3 Vacuum Infiltration Experiments with Methionine Sulphoximine - Treated Leaves

A separate experiment was performed in which Datura leaves were treated for 3 hr with a nitrate feeding solution containing 5 mM MSO (see 3.2.) prior to infiltration with a 400 μg ^{15}N. ml^{-1} nitrate feeding solution. The leaves were allowed to photosynthesise for 10, 30 and 60 mins after infiltration.

MSO appears to markedly affect ammonia assimilation into the major soluble amino compounds (Table 7). After 30 min of photosynthesis the total amount of ^{15}N incorporated into the commonest free amino acids was decreased by 99,0% and 99,7% for the high and low feeding levels respectively, compared with MSO-untreated leaves, (i.e. amino acid production was virtually eliminated). The net result of this suppression of amino acid synthesis was an accumulation of a large, heavily ^{15}N enriched ammonia pool. Stewart and Rhodes (1976) noted a similar accumulation of ammonia in MSO-treated Lemna minor.

The negligible ^{15}N-labelling of the glutamate pool following MSO treatment (Table 7) is not consistent with a biosynthetic role for GDH in Datura leaves. The possibility that GDH activity was in some way inhibited by the MSO could not be excluded so it was considered necessary to assay the enzyme in both MSO-treated and untreated leaves. Results of the assays discussed in section 4.3.1. show that both the NADH- and NADPH-dependent activities were not inhibited by MSO, the NADPH-dependent activity actually showing a 2-fold stimulation at both feeding levels

TABLE 7 : ^{15}N enrichments and distribution in the commonest free amino compounds of Datura stramonium leaves after feeding KNO_3 at 25 and 200 µg N.ml^{-1} levels for 8 hr. followed by $K^{15}NO_3$ and 5 mM methionine sulfoximine infilitration, and subsequent photosynthesis for 10, 30 and 60 min periods.

200 µg N ml^{-1} Feeding Level

	10 min			30 min			60 min		
	Enrich-ment (A%E)	Concentration in leaf (µm.gFW^{-1})	^{15}N content (µg^{15}N.gFW^{-1})	Enrich-ment (A%E)	Concentration in leaf (µm.gFW^{-1})	^{15}N content (µg^{15}N.gFW^{-1})	Enrich-ment (A%E)	Concentration in leaf (µm.gFW^{-1})	^{15}N content (µg^{15}N.gFW^{-1})
ASP	-	0,046	-	-	0,007	-	-	0,070	-
ASN	-	0,156	-	-	0,053	-	0,81	0,179	0,041
ALA	-	0,015	-	-	0,020	-	-	0,040	-
SER	-	0,268	-	-	0,121	-	0,09	0,242	0,003
THR	0,05	0,823	0,006	0,11	0,718	0,011	*	1,446	-
GLY	-	0,592	-	-	0,079	-	-	0,214	-
GLN	0,12	3,381	0,114	0,11	3,551	0,109	0,19	3,173	0,169
GLU	0,34	0,332	0,016	0,15	0,210	0,004	0,09	0,439	0,006
NH_3	2,74	20,387	7,821	7,61	20,236	21,560	11,64	15,407	25,516

25 µg N ml^{-1} Feeding Level

	10 min			30 min			60 min		
	Enrich-ment (A%E)	Concentration in leaf (µm.gFW^{-1})	^{15}N content (µg^{15}N.gFW^{-1})	Enrich-ment (A%E)	Concentration in leaf (µm.gFW^{-1})	^{15}N content (µg^{15}N.gFW^{-1})	Enrich-ment (A%E)	Concentration in leaf (µm.gFW^{-1})	^{15}N content (µg^{15}N.gFW^{-1})
ASP	-	0,009	-	-	0,019	-	-	0,043	-
ASN	-	0,021	-	-	0,039	-	0,03	0,194	0,002
ALA	-	0,003	-	-	0,009	-	-	0,026	-
SER	-	0,018	-	-	0,078	-	0,04	0,320	0,002
THR	-	0,389	-	0,05	0,767	0,005	0,08	1,820	0,020
GLY	-	0,018	-	-	0,005	-	-	0,106	-
GLN	0,04	0,647	0,007	0,07	1,266	0,025	0,17	1,037	0,049
GLU	0,27	0,159	0,006	0,11	0,221	0,003	0,07	0,255	0,003
NH_3	1,90	7,686	2,044	6,32	14,227	12,588	13,34	12,331	23,029

* Sample lost

after MSO treatment (Table 9).

These findings concerning the effect of MSO on the assimilation of newly-reduced nitrogen and on GDH activity suggest that ammonia is assimilated virtually exclusively via GS at both feeding levels. The contribution of GDH to this process, if any, is negligible when compared to the role played by GS. It is likely, then, that the pattern of ^{15}N incorporation into glutamine and glutamate in response to feeding level, is the result of the existence of separate metabolic pools of glutamine as discussed earlier (4.2.1.1.1.), and not the result of two simultaneously operative pathways of nitrogen assimilation.

It appears then, that at the high feeding level there is the synthesis of a large, storage pool of glutamine located outside the chloroplast during the pretreatment period prior to infiltration. This pool is probably not immediately available for amino acid synthesis. The existence of just such a metabolically inactive pool is illustrated in MSO-treated leaves (Table 7) which show the presence of a large, almost completely unlabelled, glutamine pool at the high feeding level even though the synthesis of this amide from newly-reduced nitrogen has been completely suppressed by MSO. Leaves induced at the lower feeding level possess a much smaller glutamine storage pool (Table 7). It is apparent that at the high feeding level the prime routing of newly-reduced ^{15}N is probably to a small, actively turning-over pool of glutamine within the chloroplast, which is actively donating amide ^{15}N to glutamate via GOGAT. The large glutamine storage pool synthesized during the pretreatment period at the high feeding level effectively masks the activity of the metabolically active pool. Such a scheme

provides an attractive explanation for the ^{15}N labelling pattern of glutamine and glutamate observed in Tables 4 and 5.

Further evidence for the crucial role played by GS in the amino acid metabolism of Datura is provided by the extensive draining of the free amino acid pools at both feeding levels, following MSO treatment (Table 7). All of the common free amino acids, with the exception of threonine which shows a one-fold increase, decreased in concentration following GS inhibition. Aspartate and alanine showed the highest percentage decrease at both feeding levels though the loss from the glutamine and glutamate pools accounted for the greatest loss in terms of total nitrogen (more than 50% of the total nitrogen of the commonest free amino acids at both feeding levels). These findings underline the central position occupied by GS in amino acid metabolism and suggest extensive channelling of glutamate and glutamine nitrogen into amino acid and protein synthesis.

4.2.1.2. Glutamine ^{15}N Feeding Experiments (Investigation of GOGAT Activity

Evidence for the participation of GOGAT is provided by an experiment (for details see 3.3.1.) where 200 $\mu g.\ ml^{-1}$ and 25 $\mu g.\ ml^{-1}$ glutamine ^{15}N (amide ^{15}N) was fed to Datura leaves via the xylem stream. The leaves were previously treated with nitrate at the same respective concentrations of nitrogen (3.2.).

The results presented in Table 8 show glutamate to be the major acceptor of the amide nitrogen of glutamine similar to the findings with pea leaves (Lewis and Pate, 1974; Bauer et al., 1977). This transfer of the amide nitrogen could be accounted for by GOGAT

TABLE 8 : ^{15}N enrichments and distribution in the commonest free amino compounds of Datura stramonium leaves following transpirational feeding of glutamine-^{15}N for 20 min and 45 min at two concentrational levels : 200 µg ^{15}N. ml^{-1} and 25 µg ^{15}N. ml^{-1}. The leaves were pretreated for 4 hr with potassium nitrate at 200 µg N. ml^{-1} and 25 µg N. ml^{-1}, respectively.

200 µg ^{15}N. ml^{-1} Feeding Level

	20 mins			45 mins		
	Enrichment A%E	Concentration in the leaf µm.gFW^{-1}	^{15}N Content µg^{15}N.gFW^{-1}	Enrichment A%E	Concentration in the leaf µm.gFW^{-1}	^{15}N Content µg^{15}N.gFW^{-1}
ASP	0,91	0,621	0,079	1,77	0,700	0,174
ASG	*			0,78	0,320	0,070
SER	0,93	1,530	0,199	1,68	1,731	0,407
GLN	4,93	5,250	7,247	6,23	5,273	9,199
GLU	1,31	1,804	0,331	2,95	2,318	0,957

25 µg ^{15}N. ml^{-1} Feeding Level

	20 mins			45 mins		
	Enrichment A%E	Concentration in the leaf µm.gFW^{-1}	^{15}N Content µg^{15}N.gFW^{-1}	Enrichment A%E	Concentration in the leaf µm.gFW^{-1}	^{15}N Content µg^{15}N.gFW^{-1}
ASP	0,38	0,282	0,015	0,84	0,208	0,024
ASG	*			0,42	0,053	0,003
SER	0,13	0,741	0,014	0,47	0,984	0,063
GLN	1,69	1,300	0,615	2,91	1,151	0,799
GLU	0,56	1,311	0,102	1,58	1,192	0,264

*Not Collected

activity or by the combined action of a glutaminase, ubiquitous in biological organisms (Prusiner, 1973), and GDH. Glutaminases catalyse the hydrolytic deamidation of L-glutamine resulting in the production of L-glutamate and ammonia. This ammonia could be re-assimilated by GDH. The demonstration in the previous section that GDH does not act in an ammonia assimilatory capacity, even in the presence of an excess of ammonia (produced by MSO treatment), precludes the operation of this route. GOGAT, therefore, appears to be the sole mediator of the amide transfer from glutamine to 2-oxoglutarate to produce glutamate in the present experiments.

Referring back to Table 8, it is apparent that at the high glutamine-^{15}N feeding level the ^{15}N content of glutamate for the 20 min and 45 min time period is $\pm$ three times that of glutamate at the lower feeding level. It is not possible, from the present experiments, to establish if this is merely the result of an increased substrate availability as glutamine-^{15}N or whether there was some stimulation of GOGAT activity during the pretreatment period.

4.2.2. The Synthesis of Alanine and Aspartate

The relatively high initial ^{15}N-labelling of aspartate and alanine (Table 6) suggests that newly-reduced ^{15}N may be incorporated directly as ammonia into these amino acids. A biosynthetic role has indeed been claimed for aspartate dehydrogenase (Santarius and Stocking, 1969) and alanine dehydrogenase (Tsukamato, 1970) catalysing the reductive amination of oxaloacetate and pyruvate, respectively. The complete suppression of ^{15}N incorporation into these amino acids following MSO

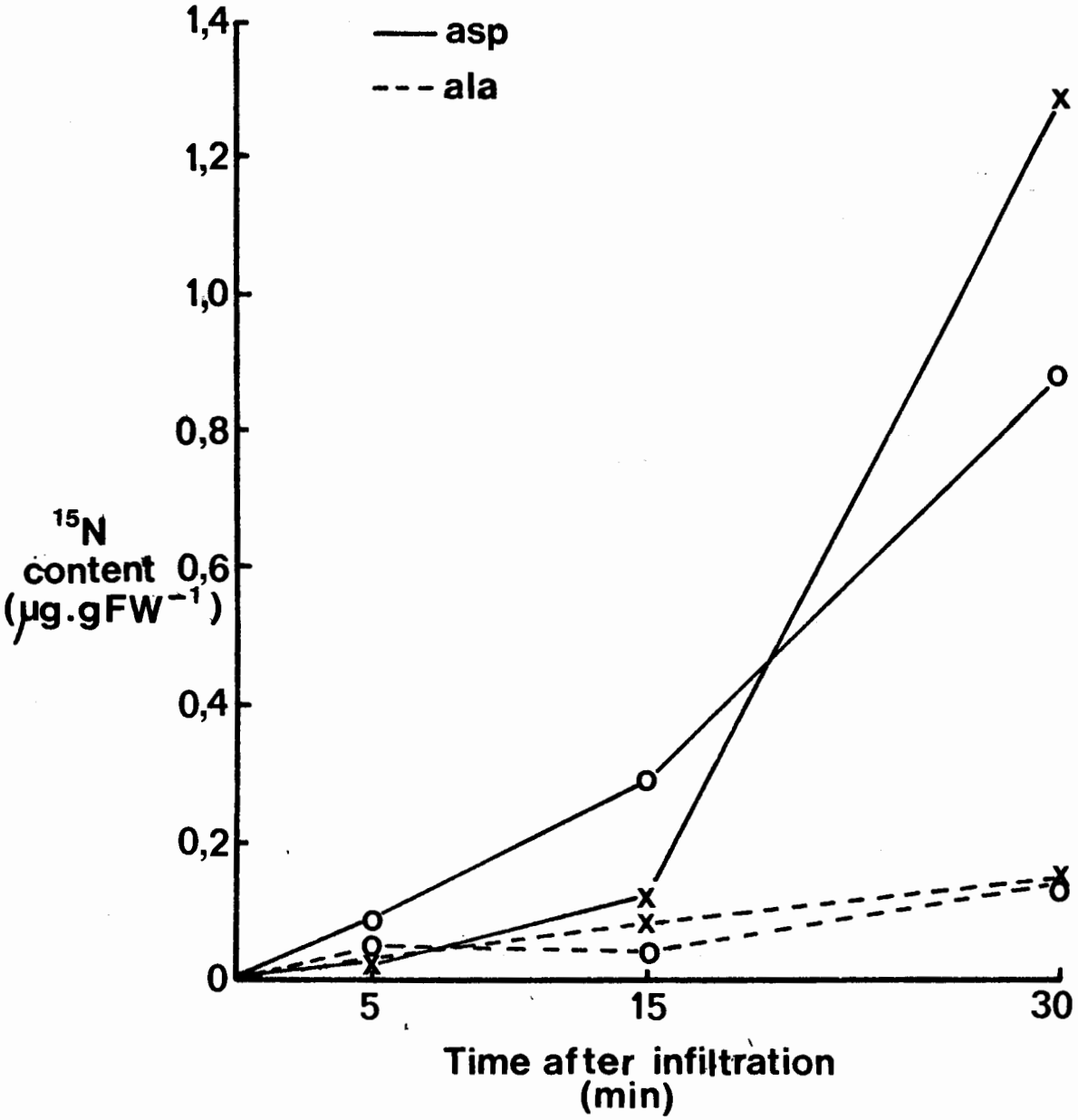

FIG. 3 : The time course of ^{15}N incorporation into the soluble aspartate (——) and alanine (----) pools of Datura leaves vacuum infiltrated with 400 µg ^{15}N. ml^{-1} nitrates and allowed to photosynthesise for 5, 15 and 30 min. Leaves were transpirationally fed either 200 µg N. ml^{-1} (0) or 25 µg N. ml^{-1} (X) for 6 hr prior to infiltration.

treatment (Table 7) precludes their participation in the assimilation of newly-reduced nitrogen. These findings, however, do not exclude the possibility of some aspartate synthesis via aspartate dehydrogenase in a compartment away from the site of formation and assimilation of ammonia produced from nitrate, as proposed by Bauer et al. (1977). An alternative mechanism whereby aspartate is formed directly from fumarate has been claimed for higher plants (Mitchell and Bidwell, 1970), although an enzyme capable of the direct amination of the double bond of fumarate has not been isolated from higher plants as of yet (Miflin and Lea, 1977).

It is most likely, then, that aspartate and alanine are synthesized by glutamate amino transfer reactions. Both glutamate-oxaloacetate aminotransferase activity (GOAT) and glutamate-pyruvate aminotransferase activity (GPAT) have been isolated from leaf chloroplasts mitochondria, peroxisomes and cytosol (Santarius and Stocking, 1969; Huang et al., 1976). GOAT and GPAT might be extremely important in the amino acid metabolism of the chloroplast in view of the fact that all common protein amino acids, with the exception of leucine, can be synthesized from aspartate and alanine in secondary amino transfer reactions in the chloroplast (Kirk and Leech, 1972).

The time course of ^{15}N incorporation into aspartate and alanine is shown in Fig. 3. The ^{15}N content of the two amino acids suggests GPAT activity is low compared to GOAT and is unaffected by feeding level. Aspartate shows no tendency to saturate during the time course of the experiment indicating a slow rate of turnover at both feeding levels. ^{15}N incorporation into aspartate shows a steep

increase after 15 mins at both feeding levels. This increase coincides with the period of maximum assimilation of ammonia into amino compounds (the ammonia pool ^{15}N content saturates after 15 mins at both feeding levels) and, therefore, is likely to be the result of an increased amino nitrogen availability after 15 mins.

4.2.3. The Synthesis of Serine and Glycine

Serine and glycine are important in the assimilation of newly-reduced nitrogen in Datura leaves being second only to glutamine and glutamate in their rate of ^{15}N accumulation (Table 6). It is apparent from Fig. 4 that accumulation of ^{15}N in serine is greater than that in glycine throughout the time course of the experiment. Serine biosynthesis via a chloroplastic phosphorylated pathway has been demonstrated in higher plants (Hanford and Davies, 1958; Cheung et al., 1968). Recent ^{14}C tracer experiments with maize (Chapman and Leech, 1976) and bean (Daley and Bidwell, 1977), however, suggest serine and phosphoserine, a proposed intermediate of the phosphorylated pathway, are not closely related metabolically. The major route for glycine and serine synthesis is thought to be via photorespiratory pathways (Tolbert, 1971; Zelitch, 1972; Challet and Ogren, 1975). Glycine, formed by the amination of glyoxyllate in the peroxisome can give rise to serine in the mitochondrion. Similarly, the amination of hydroxypyruvate in the peroxisome provides an alternative method of serine and hence glycine synthesis. Glutamate-hydroxypyruvate transaminase (Tolbert, 1973) and glutamate-glyoxyllate transaminase (Huang et al., 1976) are located exclusively in the peroxisome. The fact that ^{15}N accumulates in serine at a faster rate than in glycine

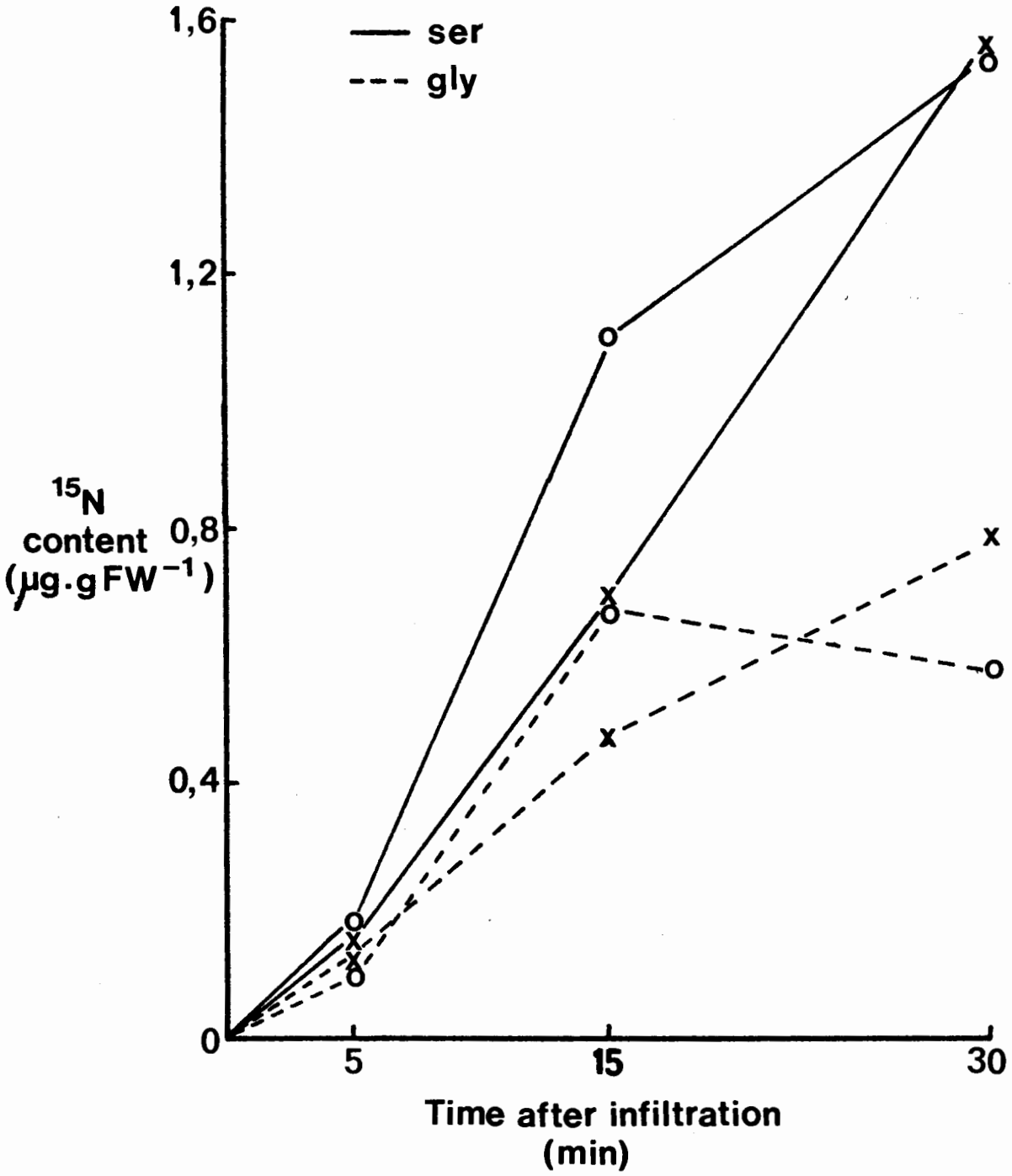

FIG. 4 : Time course of ^{15}N incorporation into the soluble serine (——) and glycine (----) pools of Datura leaves vacuum infiltrated with 400 µg ^{15}N. ml^{-1} nitrate and left to photosynthesise for 5, 15 and 30 min. Leaves were transpirationally fed either a 200 µg N. ml^{-1} (O) or a 25 µg N. ml^{-1} (X) potassium nitrate solution for 6 hr prior to infiltration.

throughout the time course of the experiment suggests the synthesis of these amino acids is primarily via hydroxypyruvate.

Glutamine-^{15}N feeding experiments indicate a substantial routing of the amide nitrogen to serine especially at the high feeding level (Table 8). This phenomenon is probably the result of the increased glutamate ^{15}N content at the high feeding level. Work by Fair, Tew and Cresswell on barley (Fair et al., 1972; 1974; Fair, 1978) has suggested that photorespiration is closely linked to nitrogen metabolism, probably being the result of the requirement for glutamate in the amination steps of photorespiration.

4.2.4. The Synthesis of Asparagine

Asparagine does not appear to be a major recipient of newly-reduced nitrogen in Datura, accounting for ±1% of the total ^{15}N assimilated into the commonest free amino compounds at both feeding levels. The incorporation of ^{15}N into asparagine appears to occur at a faster rate initially at the higher feeding level (Fig. 5), though over the 15 min to 30 min time period the rate is almost identical for both feeding levels.

Asparagine synthesis was originally considered to occur via the direct incorporation of ammonia into aspartate with the expenditure of ATP (Webster and Varner, 1955; Oaks, 1967) although attempts to demonstrate such a reaction in plant tissue, analogous to the amidation of glutamate, have failed (Lees et al., 1968; Lever and Butler, 1971). The lack of ^{15}N incorporation into asparagine following MSO treatment (Table 7) demonstrates that ammonia is not the direct substrate for

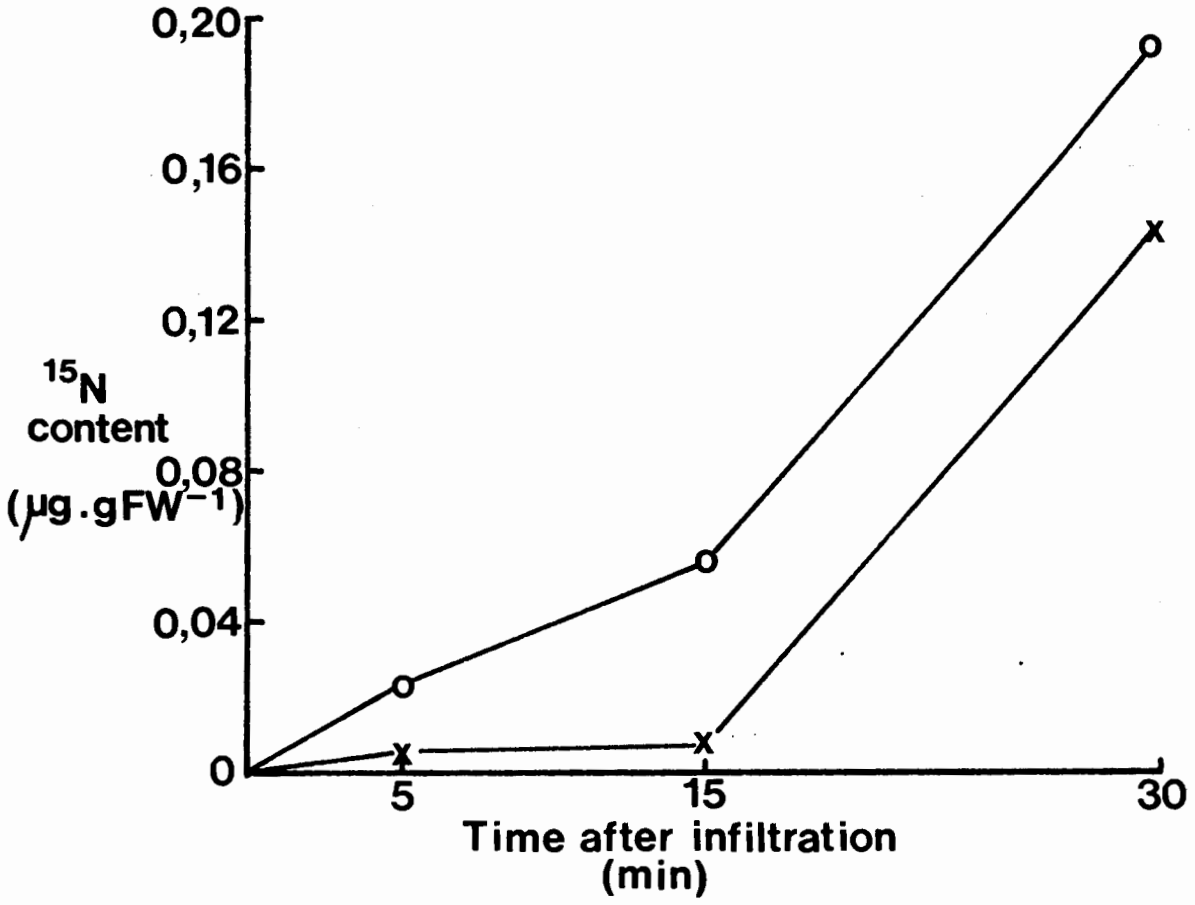

FIG. 5 : Time course of ^{15}N incorporation into the soluble asparagine pool of Datura leaves vacuum infiltrated with 400 µg ^{15}N. ml^{-1} nitrate and allowed to photosynthesise for 5, 15 and 30 min. Leaves were transpirationally fed either a 200 µg N. ml^{-1} (O) or a 25 µg N. ml^{-1} (X) potassium nitrate solution for 6 hr prior to infiltration.

asparagine synthesis. Asparagine synthesis from cyanide has been demonstrated in a variety of organisms (Blumenthal, 1963; Imada et al., 1973). This pathway, however, is not considered to be the normal route for asparagine synthesis but may be important as a cyanide detoxifier (Oaks and Johnson, 1972).

A glutamine dependent asparagine synthetase has been identified in crude extracts of lupin and soybean (Rognes, 1975; Streeter, 1973). This enzyme is believed to mediate the ATP-dependent amide transfer from glutamine to aspartate with the formation of asparagine and glutamate. Evidence supporting the involvement of glutamine in asparagine synthesis has been provided by the use of ^{14}C-labelled aspartate (Kanamori and Matsumoto, 1974) and specific inhibitors (Lea and Fowden, 1975b). The results presented in Table 8 indicate that the incorporation of the amide nitrogen of glutamine into asparagine is relatively slow, although the increased glutamine concentration at the high feeding level causes a some 20-fold enhancement of asparagine synthesis.

The origin of the carbon skeleton for asparagine synthesis is still in some doubt. ^{14}C-aspartate, the most logical precursor, is only poorly converted to asparagine when fed to Datura stramonium leaves (Nieman, 1972). However, a comparison of the time course of ^{15}N incorporation into aspartate (Fig. 3) and into asparagine (Fig. 5) reveals a parallelism between the synthesis of the two amino compounds. Both show a low initial rate of ^{15}N incorporation over the first 15 mins followed by a period with a much elevated rate of ^{15}N incorporation. The poor conversion of ^{14}C aspartate to asparagine in Datura leaves

(Nieman, 1972) can be explained if one accepts the view that asparagine biosynthesis is compartmentalised so that it is not accessible to externally-supplied aspartate.

The findings of the present experiments indicate that the synthesis of asparagine might well be closely linked metabolically to that of glutamine and aspartate.

4.2.5. The Synthesis of Threonine

In the present study the incorporation of ^{15}N into threonine was shown to be very slow (Table 5), the initial routing of ^{15}N to threonine being less than 1% of the total amount of ^{15}N incorporated into the other free amino acids. The synthesis of threonine, an intermediate of the pathway of isoleucine synthesis from aspartate (Bryan, 1976), does not appear to make a significant contribution to the primary assimilation of newly-reduced nitrogen.

One interesting point about the metabolism of threonine is the effect of MSO on the pool size of threonine in the leaf. The inclusion of MSO in the leaf feeding solution resulted in a substantial decrease in the pool sizes of all the major soluble amino compounds except threonine, which increased one-fold. It appears that in Datura leaves, threonine is probably produced outside the chloroplast by a pathway that does not involve freshly reduced nitrate nitrogen. With the slow-down of protein production owing to the shortage of other amino acids following MSO treatment, threonine would tend to accumulate.

4.3 Enzyme Assays

4.3.1. Glutamate Dehydrogenase

GDH activity was assayed in both MSO-treated and untreated leaves and roots (Section 3.8.2.3.) to establish if MSO, a known inhibitor of GS (Brenchley, 1973), also inhibited GDH. The leaves and roots were pretreated as described for the ^{15}N feeding experiments (3.2.).

The NADH-specific activity of Datura leaves is unaffected by feeding level but the NADPH-specific activity is approximately two times higher at the low feeding level (Table 9).

TABLE 9

NAD(P)H-specific activities of glutamate dehydrogenase from Datura leaves as affected by feeding level and methionine sulphoximine treatment. Activities are expressed as μmole NAD(P)H oxidised min^{-1} gFW^{-1}. Glutamate dehydrogenase activity was determined according to the method of Joy (1969).

	NADH		NADPH	
	25 $\mu g.ml^{-1}$ Feeding level	200 $\mu g.ml^{-1}$ Feeding level	25 $\mu g.ml^{-1}$ Feeding level	200 $\mu g.ml^{-1}$ Feeding level
MSO Treated	3,731	4,650	0,844	0,338
Untreated	4,013	4,294	0,281	0,113

The NADPH-dependent activity, however, represents only ±5% of the total GDH activity. MSO treatment does not affect the NADH-dependent GDH, in accordance with other reports (Brenchley, 1973), but the NADPH-specific activity showed a 2-fold stimulation after MSO treatment. This could be the result of induction by the much elevated ammonia levels following MSO treatment (Table 6). Such an interpretation is feasible if one accepts the biosynthetic role postulated for the NADP-dependent GDH of higher plant chloroplasts (Lea and Kirk, 1968; Lea and Thuruman, 1972).

The results of the ^{15}N feeding experiments, however, are not consistent with the involvement of GDH in the primary assimilation of newly synthesized ammonia.

The effect of MSO on the GDH activity of Datura roots is shown in Table 10.

TABLE 10

NAD(P)H-specific activities of GDH in methionine sulphoximine-treated and untreated Datura roots. Activities are expressed as µmole NAD(P)H oxidised . $min^{-1}.gFW^{-1}$. Glutamate dehydrogenase activity was determined according to the method of Pahlich and Joy (1973).

	NADH	NADPH
MSO Treated	1,641	0,178
Untreated	1,463	0,216

It appears that both NADH- and NADPH-dependent activities are unaffected by MSO. It is not possible to compare the relative activities in the roots and leaves because two different methods employing different extraction media were followed (3.8.1.) and because no attempt was made to optimize extraction procedures.

4.3.2. Glutamine Synthetase

Having established the key role that GS plays in the assimilation of nitrate into amino compounds (4.2.1.), it is apparent that the control of the activity of this enzyme could represent an important regulatory step in the present experiments. The central position of glutamine in nitrogen metabolism has resulted in the evolution of complex and unique regulatory mechanisms of its synthesis, well documented in micro-organisms (2.5).

GS activity was assayed in Datura leaves following a pretreatment of varying duration with either 200 μg N. ml^{-1} or 25 μg N. ml^{-1} nitrate (3.8.2.2). GS activity was determined after incubation for 30 mins during which time the reaction proceeded in a linear fashion. The effect of nitrate concentration on GS activity is summarized in Table 11.

TABLE 11

The effect of varying time periods of nitrate feeding, at 200 μg N. ml^{-1} and 25 μg N. ml^{-1} concentrational levels on the glutamine synthetase activity of Datura leaves. Glutamine synthase activity was determined according to the method of Rhodes et al. (1976).

Duration of Nitrate Feeding (hr)	Glutamine Synthetase Activity (nm. hydroxymate . hr^{-1})	
	200 μg N. ml^{-1} Feeding Level	25 μg N. ml^{-1} Feeding Level
1	54,6	51,2
2	51,6	56,3
4	57,3	60,7
6	57,0	53,4
10	56,3	58,6

It is evident that GS activity is unaffected by the level or duration of nitrate feeding. This finding indicates that the GS of Datura leaves is unaffected by its substrate, ammonia, or possibly that the difference between the amounts of ammonia produced from nitrate reduction at the high and low feeding levels (4.3.2.), was not sufficient to elicit a regulatory response. Rhodes et al. (1976) have shown the activity of GS from Lemna minor to be suppressed by an increased ammonia availability (2.5.).

4.3.3. Nitrate Reductase

Control of the activity of nitrate reductase (NR) and nitrite reductase represents another possible regulatory mechanism in the present experiments. The activity of nitrite reductase is characteristically 5-20 times that of nitrate reductase (Eg Stewart, 1972) and the low levels of nitrite compared to nitrate in plant tissues, suggest nitrite reductase has an excess capacity *in vivo* (Hewitt *et al.*, 1976). The reduction of nitrate, being the rate limiting step, is logically more suited to a regulatory role (Hageman, 1977).

The effect of the two concentrations of nitrate, 25 μg N. ml^{-1} and 200 μg N. ml^{-1}, used in the pretreatment period for ^{15}N-feeding experiments, was monitored over an eight hour time period. NR activity was determined according to the method of Bar Akiva and Sagiv (1967) as described in Section 3.8.2.1. Leaf extracts were incubated for 30 mins during which time the reaction proceeded in a linear fashion.

The development of NR activity with increasing periods of nitrate treatment is summarized in Fig. 6. The induction of NR by nitrate, known to be almost universal (Hewitt *et al.*, 1976), is well demonstrated in the present experiments. It can be seen that the synthesis of NR is complete after 4-5 hrs at both feeding levels, thereby justifying the choice of a 4-6 hr pretreatment (induction) period for the ^{15}N-feeding experiments. The fully induced activities of NR are some 3-fold higher at the 200 μg N. ml^{-1} feeding level than at the 25 μg N. ml^{-1} feeding level, presumably due to the increased flux of nitrate at the higher feeding level. NR induction has been demonstrated to be related more

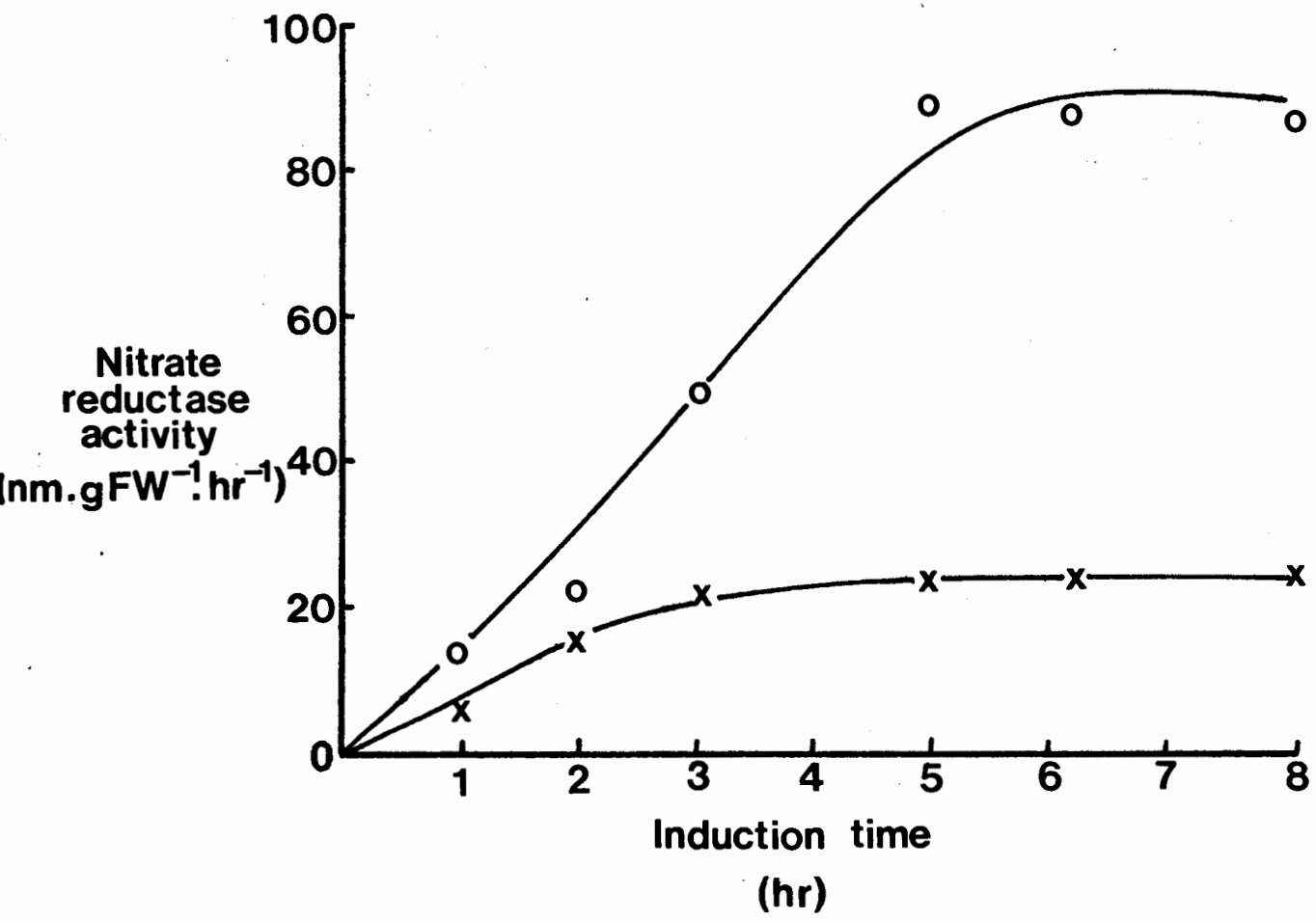

FIG. 6 : The induction of nitrate reductase actively in Datura leaves following the transpirational feeding of potassium nitrate at two concentrational levels : 200 µg N. ml^{-1} (0) and 25 µg N. ml^{-1} (X). Nitrate reductase activity was determined according to the method of Bar Akiva and Sagiv (1967).

to the nitrate entering the cells than to the nitrate that is stored (Heimer and Filner, 1971). This and other studies led to the concept of two pools of nitrate in plant cells; a large storage pool, possibly in the vacuole, and a smaller rapidly turning-over pool in the cytoplasm that may be involved in NR induction. Shaner and Boner (1976) have more recently demonstrated conclusively that the flux of nitrate in the xylem stream, as measured by the transpiration rate and nitrate content of the xylem sap, was more important by far than flux of nitrate from storage pools in inducing NR activity. In the current ^{15}N-feeding experiments and enzyme assays the flux of nitrate in the transpiration stream during the pretreatment period was ± 80 µg N. $gFW^{-1}.hr^{-1}$ and ± 10 µg N. $gFW^{-1}.hr^{-1}$ for the high and low feeding levels, respectively (calculated from the rate of water uptake by detached Datura leaves, i.e. $0,39 \pm 0,2$ ml.$gFW^{-1}.hr^{-1}$, under experimental conditions (3.2.) and the nitrate content of the feeding solution). It appears, therefore, that in the present experiments increasing the flux of nitrate eight times results in a 3-fold stimulation of NR activity.

Thus, nitrate reduction showing a sensitive response to nitrate flux constitutes the important regulatory step in the incorporation of nitrate into amino compounds in the current experiments.

4.4 The Assimilation of Nitrate-^{15}N by Root Tissue

The mechanism of ammonia assimilation by roots is less well documented than its assimilation by leaves. Both the mitochondria (Miflin, 1970; Pahlich and Joy, 1971; Joy, 1973) and plastids (Miflin, 1974) of roots are known to possess GDH. Ammonia, a substrate of the aminating reaction, induces an NADH-specific GDH in the roots of sunflower and soybean (Weissman, 1972), rice (Kanamori et al., 1972), and maize (Gasparikova et al., 1976), suggesting a possible assimilatory function for this enzyme in these plants.

Root tissue of higher plants, however, also contain a GS which has a low Km for ammonia (Kanamori and Matsumoto, 1972) and which exhibits activities some 40-fold higher than GDH (Miflin, 1974a). GOGAT has been demonstrated in pea roots (Fowler et al., 1974; Miflin and Lea, 1975) establishing the potential for ammonia assimilation via the combined action of GS and GOGAT in roots. These enzymes have been detected in the symbiotic nodules of legumes but further work is required in this field to confirm the pathway of nitrogen flux in such associations (Miflin and Lea, 1976b).

Studies involving the feeding of ^{15}N-substrates to roots provide further evidence concerning the pathway of nitrogen assimilation by root tissue. Ivanko and Ingversen (1971), after feeding ^{15}N-nitrate to maize roots for 6 hrs, demonstrated a routing of newly-reduced nitrogen principally to glutamate. The authors interpret these results as evidence for the operation of the GDH pathway. These experiments, however, were performed over a long time period and therefore

do not necessarily reflect the initial pattern of ^{15}N assimilation. In contrast, Cocking and Yemm (1961) found both the amino and amido nitrogen of glutamine to have a much higher ^{15}N enrichment than glutamate after ^{15}N-ammonium feeding to barley roots for 1 hr. More detailed time course studies have been performed on rice roots where in both ^{15}N-ammonium fed roots (Yoneyama and Kumazawa, 1974; Arima and Kumazawa, 1975) and ^{15}N-nitrate fed roots (Yoneyama and Kumazawa, 1975) glutamine was initially the most heavily labelled amino compound. Corn roots show a similar pattern of ^{15}N-incorporation with the amide nitrogen of glutamine containing the highest ^{15}N enrichment after both nitrate and ammonium feeding (Yoneyama et al., 1977). These findings are consistent with the operation of the GS/GOGAT pathway.

In view of the lack of conclusive evidence favouring any one pathway for nitrogen assimilation by roots, it was considered necessary to investigate this phenomenon in the roots of Datura. Xylem sap analyses (4.1.) suggest that Datura roots are capable of nitrate reduction and ammonia assimilation.

4.4.1. Infiltration of Nitrate-^{15}N

Datura roots were pressure infiltrated with 400 ug $^{15}N.ml^{-1}$ nitrate following a pretreatment period in either an MSO-free 100 μg $N.ml^{-1}$ nitrate nutrient solution or an MSO-containing 100 μg $N.ml^{-1}$ nitrate nutrient solution (see Tables 1 and 2 for details of the nutrient solution).

The incorporation of ^{15}N into the commonest free amino acids of Datura roots in the absence of MSO treatment is shown in Table 12.

TABLE 12 : ^{15}N enrichments and distribution in the commonest free amino compounds of Datura stramonium roots after pressure infiltration with 400 µg $^{15}N.ml^{-1}$ potassium nitrate. Roots were allowed to metabolize for 5, 15 and 30 mins after infiltration.

	5 min			15 min			30 min		
	Enrichment (A%E)	Concentration in the leaf (µm.gFW^{-1})	^{15}N content (µg^{15}N.gFW^{-1})	Enrichment (A%E)	Concentration in the leaf (µm.gFW^{-1})	^{15}N content (µg^{15}N.gFW^{-1})	Enrichment (A%E)	Concentration in the leaf (µm.gFW^{-1})	^{15}N content (µg^{15}N.gFW^{-1})
ASP	0,16	0,148	0,003	0,40	0,127	0,007	1,13	0,116	0,018
ASN	0	0,063	0	0,20	0,072	0,004	0,23	0,061	0,004
ALA	0,23	0,077	0,001	0,62	0,163	0,014	0,77	0,084	0,009
SER	*	0,072	-	0,19	0,104	0,003	0,30	0,063	0,003
THR	0	0,037	0	0,09	0,047	0	0,25	0,033	0
GLY	0	0,011	0	0,08	0,027	0	0,15	0,010	0
GLN	1,56	0,342	0,152	3,18	0,325	0,290	4,13	0,296	0,347
GLU	0,50	0,268	0,019	1,52	0,319	0,072	2,17	0,257	0,078
NH_3	2,65	0,482	0,179	3,02	0,481	0,204	3,88	0,423	0,230

*Sample lost

The pattern of labelling obtained is similar to that reported for rice roots (Yoneyama and Kumazawa, 1975) and corn roots (Yoneyama et al., 1977) with glutamine, glutamate, aspartate and alanine being the primary recipients of newly-reduced nitrate-^{15}N. Threonine and glycine were unlabelled during the time course of the experiment and ^{15}N label appeared only slowly in asparagine and serine (Table 12).

Both the enrichment and ^{15}N content of glutamine reveal its importance as an initial acceptor of newly-reduced nitrogen. In this respect the labelling pattern of the roots (Table 12) differs from the leaves (Table 5) where glutamate had consistently the highest enrichment but not necessarily ^{15}N content. In the root the ^{15}N content of glutamine is initially an order of magnitude higher than in any other amino compound. Ammonia, however, has a slightly higher ^{15}N content than glutamine initially but rapidly saturates as the ^{15}N content of glutamine continues to increase (Fig. 7). The similar rates of ^{15}N incorporation into glutamine and ammonia over the first 5 mins after infiltration (Fig. 7), suggests that the two processes are closely linked metabolically, i.e., that the synthesis of glutamine is probably the major mechanism of ammonia assimilation into amino compounds. The ^{15}N content of the glutamate pool, being considerably lower than that of glutamine, does not parallel the ammonia curve. This suggests that the synthesis of glutamate is not directly affected by the availability of ammonia although the participation of GDH cannot be ruled out.

It is interesting to note that the glutamate ^{15}N content saturates after 15 min at a low enrichment value, indicating the

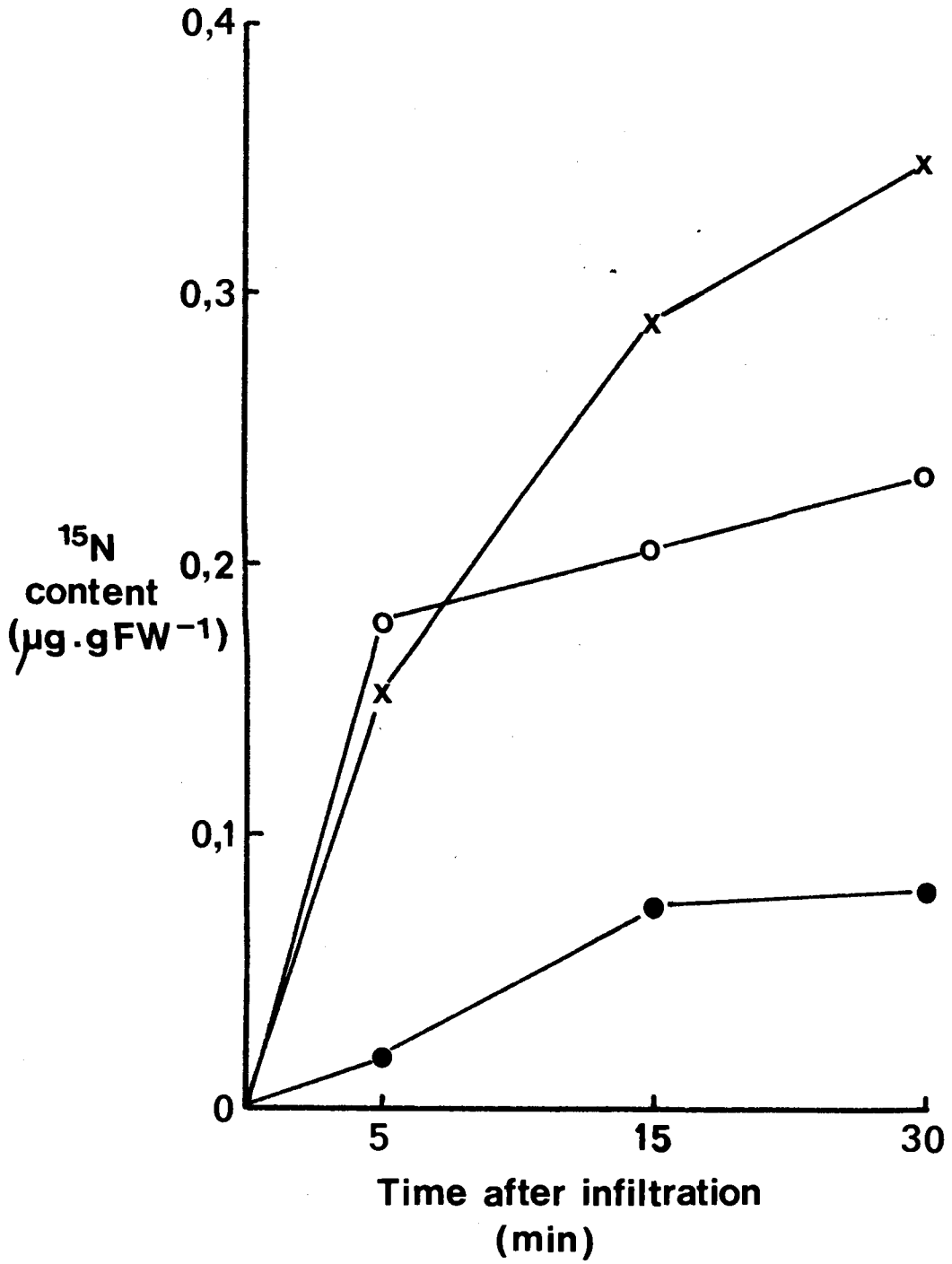

FIG. 7 : ^{15}N content of the glutamine (0—0), glutamate (•—•) and ammonia (X——X) pools of Datura roots at 5, 15 and 30 min after infiltration with 400 µg ^{15}N. ml^{-1} nitrate. Roots were pretreated in liquid culture with a 100 µg N. ml^{-1} nitrate nutrient solution for 2 days prior to infiltration.

existence of at least two pools of glutamate: a small actively turning-over pool involved in the primary assimilation of newly-reduced ^{15}N; and a larger storage pool.

The rate of ^{15}N incorporation into the soluble amino compounds of the roots appears to be much slower than in the leaves. The much decreased relative rate of ^{15}N labelling of serine and the absence of ^{15}N incorporation into glycine throughout the time course of the experiment, is consistent with their synthesis from photosynthetic intermediates. The root, although showing a limited capacity for serine synthesis, probably relies on the leaf for its supply of serine and glycine. Lewis (1975) has shown serine to be a major translocating medium of reduced nitrogen and photosynthetically fixed carbon from Datura stramonium leaves.

Threonine and asparagine (Table 12) are not important in the initial metabolism of newly-reduced nitrogen in the roots, as was the case for the leaves. Aspartate and alanine do not appear to be synthesized from ammonia produced by nitrate reduction (4.4.2.) and are therefore, most probably, the initial products of glutamate aminotransferase reactions (as occurs in the leaves).

4.4.2. Nitrate-^{15}N Infiltration with Methionine Sulphoximine-Treated Roots

To test the dependence of ammonia assimilation on GS and the possible contribution of GDH in this process, roots were pretreated with MSO (3.2.). The inhibitor had a similar effect on ^{15}N assimilation in the roots (Table 11) as was reported earlier for the leaves. Ammonia

TABLE 13 : ^{15}N enrichments and distribution in the commonest free amino compounds of Datura stramonium roots after pressure infiltration with 400 µg $^{15}N.ml^{-1}$ potassium nitrate. The roots were treated for 3 hr with 7 mM methionine sulphoximine prior to infiltration. The roots were allowed to metabolize for 5, 15 and 30 min after infiltration.

	5 min			15 min			30 min		
	Enrichment (A%E)	Concentration in the leaf ($\mu m.gFW^{-1}$)	^{15}N content ($ug^{15}N.gFW^{-1}$)	Enrichment (A%E)	Concentration in the leaf ($\mu m.gFW^{-1}$)	^{15}N content ($ug^{15}N.gFW^{-1}$)	Enrichment (A%E)	Concentration in the leaf ($\mu m.gFW^{-1}$)	^{15}N content ($\mu g^{15}N.gFW^{-1}$)
ASP	0,03	0,230	0	0,04	0,217	0	0,50	0,280	0,002
ASN	0	0,141	0	0	0,154	0	0,02	0,144	0
ALA	0	0,043	0	0	0,187	0	0,01	0,173	0
SER	0,03	0,129	0	0,02	0,116	0	0	0,190	0
THR	0,04	0,242	0	0,04	0,146	0	0,01	0,236	0
GLY	0	0,021	0	0,04	0,036	0	0,02	0,049	0
GLN	0,09	0,308	0,008	0,11	0,231	0,007	0,06	0,249	0,004
GLU	0,02	0,307	0	0,07	0,459	0,004	0,08	0,480	0,005
NH_3	0,71	2,724	0,270	2,03	2,976	0,845	3,59	3,650	1,836

assimilation was completely suppressed, with the resultant accumulation of ^{15}N in large ammonia pools. The accumulation of ^{15}N in the ammonia pool following MSO-treatment proceeded in a more or less exponential fashion. This indicates that the tendency for the ^{15}N content of the ammonia pool to saturate in the MSO-free roots (Fig. 7) is the result of the demand on this substrate for glutamine synthesis.

The small amount of ^{15}N incorporation into glutamine initially following MSO-treatment is probably the result of incomplete inhibition of glutamine synthetase by the analogue. The complete lack of ^{15}N incorporation, initially, into glutamate, alanine and aspartate does not support the participation of dehydrogenase enzymes in the primary assimilation of ammonia produced from nitrate reduction in the roots. GDH activity was assayed in the roots following similar pretreatment conditions as described for ^{15}N feeding experiments (3.8.2.3.). Both the NADH- and NADPH-dependent activities were found to be unaffected by MSO treatment (Table 10). The finding negates the possibility that glutamate dehydrogenase could act in a primary ammonia assimilatory role in the presence of excess ammonia in the roots. It also appears unlikely that glutamate dehydrogenase could be acting in sequence with a glutaminase to mediate the transfer of the amide nitrogen of glutamine to glutamate. These results demonstrate the probable exclusive operation of the glutamine synthetase/glutamate synthetase in the primary assimilation of newly-reduced ^{15}N in Datura roots.

Root tissue shows one marked difference to leaf tissue in its response to MSO. In the roots a pretreatment with MSO causes an increase in the soluble amino compound pool sizes (Table 12), whereas in the leaf

all amino compounds, except threonine, decreased in concentration following a similar treatment (4.2.1.1.3.). Only glutamine showed a slight decrease in the roots. The increase in the soluble amino compounds in Datura roots is most likely a result of the mobilization of storage protein. Nitrogen limitation is known to increase protein degradation (Huffaker and Peterson, 1974; Humphrey and Davies, 1976). It has subsequently been demonstrated that although nitrogen deprivation enhances protein turnover, the partition of amino acids between protein synthesis and amino acid metabolism is relatively constant (Davies and Humphrey, 1978). This results in an increase in the total amount of amino acid recycling, a phenomenon induced in Datura roots by MSO.

CHAPTER 5

CONCLUSIONS

The GS/GOGAT pathway is apparently the exclusive route for 2-amino production from inorganic nitrogen in the leaves of Datura, irrespective of nitrogen availability. GDH activity (mainly NADH-specific) does not appear to contribute significantly to the assimilation of ammonia produced from nitrate reduction, even under conditions of a large excess of ammonia. The in vivo role of both the NADH- and NADPH-specific enzyme thus appears to be predominantly catabolic. GS activity is unaffected by the two concentrational levels of nitrate used in the present experimentation. NR activity, therefore, being substrate inducible, constitutes a more important regulatory step than GS activity in the assimilation of nitrate into amino compounds by Datura leaves.

It appears that in Datura leaves, newly-reduced nitrogen is assimilated first into a small, rapidly saturating pool of glutamine which is actively donating amide nitrogen to glutamate via a GOGAT mediated reaction. A supply of nitrogen in excess of the leaf's current requirements, apparently results in extensive export of glutamine from this pool (probably in the chloroplast) to a larger storage pool (probably in the cytosol) which appears to be not immediately available for amino acid biosynthesis.

The major soluble leaf amino compounds, other than glutamate and glutamine, which are important in the early metabolism of newly-reduced nitrogen include serine, glycine, aspartate and alanine. All are most

likely synthesized in glutamate amino transfer reactions.

The root, although exporting mainly inorganic nitrogen to the leaf, is capable of nitrate reduction and ammonia assimilation. The assimilation of newly-reduced nitrogen into amino compounds by the roots appears to proceed primarily via the GS/GOGAT pathway, as is the case for the leaves. GDH activity (mainly NADH-specific) is unaffected by cellular ammonia concentrations and is probably involved in glutamate catabolism rather than assimilation, in vivo.

The root exhibits a lower rate of metabolism of nitrogen produced from nitrate reduction than the leaves. Besides glutamine and glutamate, the only other amino compounds that contribute significantly to the early metabolism of newly-reduced nitrogen in the root are aspartate and alanine.

REFERENCES

ANDERSON J. W. and DONE J. (1977). A polarographic study of glutamate synthase activity in isolated chloroplasts. Plant Physiol. 60, 354-359.

ARIMA Y. and KUMAZAWA K. (1975). A kinetic study of amide and amino acid synthesis in rice seedling roots fed with ^{15}N-labelled ammonium. J. Sci. Soil Manure, Japan 46, 355-361.

ATKIN G. E. and FERDINAND W. (1970). Accelerated amino acid analysis. Studies on the use of Lithium citrate buffers and the effect of n-propanol in the analysis of physiological fluids and protein hydrolyzates. Anal. Biochem. 38, 313-330.

ATKINSON D. E. (1969). Regulation of enzyme function. Ann. Rev. Microbiol. 23, 47-64.

AVRON M. (1960). Photophosphorylation by swisschard chloroplasts. Biochem. Biophys. Acta. 40, 257-272.

BAHR J. T. and JENSEN R. G. (1974). Ribulose diphosphate carboxylase from freshly ruptured spinach chloroplasts having an *in vivo* Km (CO_2). Pl. Physiol. Wash. 53, 39-44.

BAR AKIVA A. and SAGIV J. (1967). Nitrate reductase in the citrus plants. Properties assay conditions and distribution within the plant. Physiol. Plant 20, 500-507.

BARRATT R. W. (1963). Effect of environmental conditions on the NADP-specific glutamic acid dehydrogenase in *Neurospora crassa*. J. Gen. Microbiol. 33, 33-42.

BASSHAM J. A. and KIRK M. (1964). Photosynthesis of amino acids. Biochem. Biophys. Acta. 90, 553-562.

BAUER A., JOY K. W. and URQUHART A. A. (1977). Amino acid metabolism of pea leaves. Labelling studies on utilization of amides. Plant Physiol. 59, 920-924.

BLUMENTHAL-GOLDSMIDT S., BUTLER G. W. and CONN E. E. (1963). Incorporation of hydrocyanic acid labelled with carbon-14 into asparagine in seedlings. Nature 197, 718-719.

BASSHAM J. A. and JENSEN R. G. (1967). Photosynthesis of carbon compounds. In: A. San Pietro, F. A. Green and T.J. Army, eds., *Harvesting the Sun*, *Photosynthesis in Plant Life*. Academic Press, New York and London pp. 79-110.

BEEVERS L. and HAGEMAN R.H. (1969). Nitrate reduction in higher plants. Ann. Rev. Plant Physiol. 20, 495-522.

BEEVERS L. and STOREY R. (1976). Glutamate synthase in developing cotyledons of Pisum sativum. Plant Physiol. 57, 862-866.

BOLLARD E. G. (1957). Translocation of organic nitrogen in the xylem. Aust. J. Biol. Sci. 10, 292-301.

BOTTOMLEY W. (1970). Some effects of Triton X-100 on pea etioplasts. Plant Physiol. 46, 437-441.

BOWES G., OGREN W. L. and HAGEMAN R. H. (1971). Phosphoglycolate production catalyzed by ribulose diphosphate carboxylase. Biochem. Biophys. Res. Commun. 45, 716-722.

BOWLING D. J. F., MACKLON A. E. S. and SPANSWICK R.M. (1966). Active and passive transport of the major nutrient ions across the root of Ricinus communis. J. Exp. Bot. 17, 410-416.

BRENCHLEY J. E. (1973). The effect of methionine sulfoximine and methionine sulfone on glutamate synthesis in Klebsiella aerogenes. J. Bacteriol. 114, 666-673.

BROWN D. H. and HASLETT B. (1972). The changes of glutamate dehydrogenase activity following illumination of etiolated barley seedlings. Planta (Berl.) 103, 129-133.

BROWN C. M. and JOHNSON B. (1970). Influence of the concentration of glucose and galactose on the physiology of Saccharomyces cerevisial in continuous culture. J. Gen. Microbiol. 64, 279-287.

BROWN C. M., McDONALD-BROWN D. S. and MEERS J. L. (1974). Physiological aspects of microbial inorganic nitrogen metabolism. Adv. Microbial Physiol. 11, 1-52.

BROWN C. M., McDONALD-BROWN D. S. and STANLEY (1972). Inorganic nitrogen metabolism in marine bacteria : nitrogen assimilation in some marine pseudomonads. J. Mar. Biol. Ass. U.K. 52, 793-804.

BRYAN J. K. (1976). Amino acid biosynthesis and its regulation in Plant Biochemistry 3rd edition. Edited by J. Bonner and J. E. Varner. pp. 525-557. Academic Press New York, San Francisco, London.

BULEN W. A. (1956). The isolation and characterization of glutamic dehydrogenase from corn leaves. Archs. Biochem. Biophys. 62, 173-183.

BURN V. J., TURNER P. R. and BROWN C. M. (1974). Aspects of inorganic nitrogen assimilation in yeasts. Antonie van Leeuwenhoek 40, 93-102.

COCKING E. C. and YEMM E. W. (1961). Synthesis of amino acids and proteins in barley seedlings. New Phytol. 60, 103-116.

CHAPMAN D. J. and LEECH R. M. (1976). Phosphoserine as an early product of photosynthesis in isolated chloroplasts and leaves of Zea mays seedlings. FEBS Lett. 68, 160-164.

CHEUNG G. P., ROSENBLUM I. and SALLACH H. J. (1968). Comparative studies of enzymes related to serine metabolism in higher plants. Plant Physiol. 43, 1813-1822.

CHOLLET R. and OGREN W. L. (1975). Regulation of photorespiration in C_3 and C_4 species. Bot. Rev. 41, 138-168.

CHOU K. H. and SPLITTSTOESSER W. E. (1972). Glutamate dehydrogenase from pumpkin cotyledons. Plant Physiol. 49, 550-554.

DAINTY R. H. (1972). Glutamate biosynthesis in Clostridium pasteurianum and its significance to nitrogen metabolism. Biochem. J. 126, 1055-1056.

DALEY L. S. and BIDWELL R. G. S. (1977). Phosphoserine and phosphohydroxypyruvic acid. Evidence for their role as early intermediates in photosynthesis. Plant Physiol. 60, 109-114.

DALLING M. J., TOLBERT N. E. and HAGEMAN R. H. (1972). Intracellular location of nitrate reductase and nitrite reductase. In spinach and tobacco leaves. Biochem. Biophys. Acta. 283, 505-512.

DAVIES D. D. and HUMPHREY T. J. (1978). Amino acid recycling in relation to protein turnover. Plant Physiol. 61, 54-58.

DAVIES D. D. and A. N. TEIXEIRA (1975). The synthesis of glutamate and control of glutamate dehydrogenase in pea mitochondria. Phytochemistry 14, 647-656.

DELWICHE C. C. (1977). Energy relations in the global nitrogen cycle. Ambio 6, 106-111.

DENTON M. D. and GINSBURG A. (1970). Conformational changes in glutamine synthetase from Escherichia coli. I. the binding of Mn^{++} in relation to some aspects of the enzyme structure and activity. Biochemistry 8, 1714-1725.

DENTON M. D. and GINSBURG A. (1970). Some characteristics of the binding of substrates to Escherichia coli. Biochemistry 9, 617-632.

DENNEN D. W. and NIEDERPRUEM D. J. (1967). Regulation of glutamate dehydrogenases during morphogenesis of Schizophyllum commune. J. Bacterial. 93, 904-913.

DEUEL T. F. and STADTMAN E. R. (1970). Some kinetic properties of Bacillis subtillis glutamine synthetase. J. Biol. Chem. 245, 5206-5213.

DIXON R. E. (1976). Xylem translocation of ^{14}C-labelled amino acids from roots of Populus deltoides seedlings. Plant Physiol. 57 (Suppl.).

DOUGALL D. K. (1974). Evidence for the presence of glutamate synthase in extracts of carrot cell cultures. Biochem. Biophys. Res. Commun. 58, 639-646.

DOUGALL D. K. and BLOCH J. (1976). A survey of the presence of glutamate synthase in plant cell suspension cultures. Can. J. Bot. 54, 2924-2927.

DUKE S. H. and KOUKKARI W. L. (1977). Glutamate dehydrogenase activity in Lemna perpusilla 6746 : The effects of sucrose, glucose and fructose addition to growth media. Physiol. Plant. 39, 67-72.

DUKE S. H., KOUKKARI W. L. and SOULTEN T. K. (1975). Glutamate dehydrogenase activity in roots distribution in a seedling and storage root and the effects of red and far red illuminations. Physiol. Plant 34, 8-13.

ELMERICH C. and AUBERT J. P. (1971). Role of glutamine synthetase in the repression of bacterial sporulation. Biochem. Biophys. Res. Comm. 46, 892-898.

EMKE A. and HARTMAN T. (1976). Properties of glutamate dehydrogenase from Lemna minor. Phytochemistry 15, 1611-1617.

EPPLEY R. W. and ROGERS J. N. (1970). Inorganic nitrogen assimilation of Ditylium brightwellii, a marine plankton diatom. J. Phycol. 6, 344-352.

FAIR P. (1978). An investigation into the effect of varying nitrogen regimes on the abundance of peroxisomes and activities of nitrate reductase and catalase in barley (Hordeum vulgare L.) and maize Zea mays L.). Ann. Bot. 42, 101-107.

FAIR P., TEW J. and CRESSWELL C. (1972). The effect of age and leaf position on carbon dioxide compensation point, and potential photosynthetic capacity, photorespiration and nitrate assimilation in Hordeum vulgare L. J. S. Afr. Bot. 38, 81-95.

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.

FAUST H. (1967). Probenchemie ^{15}N-markierter Stickstoffuer-bindungen im Mikro-bis Nanomolbereich fur die emissionsspektrometrische Isotopenanalyse. Isotopenpraxis 3, 100-103.

FAWOLE M. O. and BOULTER D. (1977). Purification and properties of glutamate dehydrogenase from Vigna unguiculata (L) Walp. Planta (Berl.) 134, 97-102.

FERGUSON A. R. and SIMS A. P. (1971). Inactivation in vivo of glutamine synthetase and NAD-specific glutamate dehydrogenase: Its role in the regulation of glutamine synthesis in yeasts. J. Gen. Microbiol. 69, 423-427.

FERGUSON A. R. and SIMS A. P. (1974a). The regulation of glutamine metabolism in Candida utilis: The role of glutamine in the control of glutamine synthetase. J. Gen. Microbiol. 80, 159-171.

FERGUSON A. R. and SIMS A. P. (1974b). The regulation of glutamine metabolism in Candida utilis: The inactivation of glutamine synthetase. J. Gen. Microbiol. 80, 173-185.

FOWLER M. W., JESSUP W. and SARKISSIAN G. S. (1974). Glutamate synthase type activity in higher plants. FEBBS Lett. 46, 340-342.

FRIEDEN C. (1965). Glutamate dehydrogenase. Survey of purine nucleotide and other effects on the enzyme from various sources. J. Biol. Chem. 240, 2028-2035.

GANCEDO C. and HOLZER H. (1968). Enzyme inactivation of glutamine synthetase in Enterobacteriaceae. Eur. J. Biochem. 40, 190-192.

GARLAND W. J. and DENIS D. T. (1977). Steady state kinetics of glutamate dehydrogenase from Pisum sativum L. mitochondria. Arc. Biochem. Biophys. 182, 614-625.

GASPARIKOVA O., PSENAKOVA T. and NIZNANSKA A. (1976). Influence of various nitrogen sources on the activity of nitrate and nitrite reductases and glutamate dehydrogenase in Zea mays roots. Biologia (Bratislavia) 31, 527-535.

GAYLER K. R. and MORGAN W. R. (1976). A NADP-dependent glutamate dehydrogenase in chloroplasts from the marine alga Caulerpa simpliciuscula. Plant Physiol. 58, 283-287.

GINSBURG A. (1967). Conformational changes in glutamine synthetase from Escherichia coli. II. Some characteristics of the equilibrium binding of feedback inhibitors of the enzyme. Biochemistry, 8, 1726-1732.

GIVAN C. V. (1975). Light dependent synthesis of glutamine in pea chloroplast preparations. Planta (Berl.) 122, 281-291.

GIVAN C. V. (1976). Glutamine synthesis and its relation to photophosphorylation in Pisum chloroplasts. Plant Physiol. 57, 623-627.

GIVAN C. V., GIVAN A. L. and LEECH R. M. (1970). Photoreduction of ketoglutarate to glutamate by Vicia faba chloroplasts. Plant Physiol. 45, 624-630.

GOOD N. E. (1960). Activation of the Hill Reaction by amines. Biochem. Biophys. Acta. 40, 502-517.

GROVER A. K. and KAPOOR M. (1973). Studies on the regulation, sub-unit structure, and some properties of NAD-specific glutamate dehydrogenase of Neurospora. J. Exp. Bot. 24, 847-861.

HAGEMAN R. H. (1977). Integration of nitrogen assimilation in relation to yield. Second Long Ashton Symposium In Press.

HANFORD J. and DAVIES D. D. (1958). Formation of phosphoserine from 3-phosphoglycerate in higher plants. Nature 182, 532-533.

HAYSTEAD A. (1973). Glutamine synthetase in the chloroplasts Vicia faba. Planta (Berl.) 111, 271-274.

HEBER U. (1974). Metabolite exchange between the chloroplast and cytoplasm. Ann. Rev. Pl. Physiol. 25, 393-421.

HEBER U. W. and SANTARIUS K. A. (1965). Compartmentation and reduction of pyridine nucleotides in relation to photosynthesis. Biochem. Biophys. Acta. 109, 390-408.

HEIMER Y. M. and FILNER P. (1971). Regulation of the nitrate assimilation pathway in cultured tobacco cells. III The nitrate uptake system. Biochem. Biophys. Acta. 230, 362-372.

HEWITT E. J., HUCKLESBURY D. P. and NOTTON B. A. (1976). Nitrate Metabolism in Plant Biochemistry 3rd edition. Edited by J. Bonner and J. E. Varner. pp 633-672. Academic Press, New York, San Francisco, London.

HILLER A., PLAZIN J. and VAN SLYKE D. D. (1948). A study of conditions for Kjeldahl determination of nitrogen in proteins. J. Biol. Chem. 176, 1401-1420.

HUANG A. H. C., LIU K. D. F. and YOULE R. J. (1976). Organelle-specific isozymes of aspartate a-ketoglutarate transaminase in spinach leaves. Plant Physiol. 58, 110-113.

HUBBARD J. S. and STADTMAN E. R. (1967). Regulation of glutamine synthetase II Patterns of feedback inhibition in micro-organisms. J. Bacteriol. 93, 1045-1055.

HUFFAKER R. C. and PETERSON L. W. (1974). Protein turnover in plants and possible means of its regulation. Ann. Rev. Plant. Physiol. 25, 363-392.

HUMPHREY T. T. and DAVIES D. D. (1976). A new method for measurement of protein turnover. Biochem. J. 148, 119-127.

HUNT J. B. and GINSBURG A. (1972). Some kinetics of the interaction of divalent cations with glutamine synthetase from Escherichia coli. Metal ion induced conformational changes. Biochemistry 11, 3723-3735.

HWANG T. C. and SOULEN T. K. (1972). Properties of glutamate dehydrogenase from Hydrogenomonas facilis. Plant Physiol. 49 (Abs 199).

IMADA A., IGARASI S., NAKAHAMA K. and ISONO M. (1973). Asparaginase and glutaminase activities of micro-organisms. J. Gen. Microbiol. 76, 85-99.

INGVERSON J. and IVANKO S. (1971). Investigation on the assimilation of nitrogen by maize roots and the transport of some major nitrogen compounds by xylem sap. II Incorporation of taken-up nitrogen into free amino acids and proteins of maize roots. Physiol. Plant. 24, 199-204.

ITO O. and KUMAZAWA K. (1976). Nitrogen assimilation in sunflower leaves and upward and downward transport of nitrogen. Soil Sci. Plant Nutr. 22, 181-189.

IVANKO S. and INGVERSEN J. (1971). Investigation on the assimilation of nitrogen by maize roots and the transport of some major nitrogen compounds by xylem sap. II Transport of nitrogen compounds by xylem sap. Physiol. Plant. 24, 355-362.

JOHANSSON B. C. and GEST H. (1976). Inorganic nitrogen assimilation by the photosynthetic bacterium Rhodopseudomonas capsulata. J. Bacteriol. 128, 683-688.

JONES R. W. and SHEARD R. W. (1972). Nitrate reductase activity: phytochrome mediation in etiolated peas. Nature 238, 221-222.

JOY K. W. (1969). Nitrogen metabolism of Lemna minor. II Enzymes of nitrate assimilation and some aspects of their regulation. Plant Physiol. 44, 849-853.

JOY K. W. (1971). Glutamate dehydrogenase changes in Lemna not due to enzyme induction. Plant Physiol. 47, 445-446.

JOY K. W. (1973). Control of glutamate dehydrogenase from Pisum sativum roots. Phytochemistry 12, 1031-1040.

KAMEN M. D. (1957). Isotopic tracers in biology. Academic Press Inc. New York.

KANAZAWA T., KIRK M. R. and BASSHAM J. A. (1970). Regulatory effects of ammonia on carbon metabolism in photosynthesizing Chlorella pyrenoidosa. Biochem. Biophys. Acta. 205, 401-408.

KANAMORI T., KONISHI S. and TAKAHASHI E. (1972). Inducible formation of glutamate dehydrogenase in rice plant roots by addition of ammonia to the media. Physiol. Plant. 26, 1-6.

KANAMORI T. and MATSUMOTO H. (1972). Glutamine synthetase from rice plant roots. Arch. Biochem. Biophys. 125, 404-412.

KANAMORI T. and MATSUMOTO H. (1974). Asparagine biosynthesis by Oryza sativa seedlings. Phytochemistry 13, 1407-1412.

KAPOOR M. and GROVER A. K. (1970). Catabolite-controlled regulation of glutamate dehydrogenase of Neurospora crassa. Can. J. Microbiol. 16, 33-40.

KEDENBURG C. P. (1971). A lithium buffer system for accelerated single column amino acid analysis in physiological fluids. Analyt. Biochem. 40, 35-42.

KENNEDY I. R. (1966a). Primary products of symbiotic nitrogen fixation. I. Short-term exposure of Seradella nodules to $^{15}N_2$. Biochem. Biophys. Acta. 130, 285-303.

KENNEDY I.R. (1966b). Primary products of symbiotic nitrogen fixation II Pulse-labelling of Seradella nodules with $^{15}N_2$. Biochem. Biophys. Acta. 130, 295-303.

KINGDON H. S. (1974). Feedback inhibition of glutamine synthetase from green pea seeds. Arch. Biochem. 163, 429-431.

KINGDON H. S. and STADTMAN E. R. (1967b). Regulation of glutamine synthetase. X. Effect of growth conditions on the susceptibility of E. coli glutamine synthetase to feedback inhibition. J. Bacteriol. 94, 949-958.

KINGDON H. S., SHAPIRO B. M. and STADTMAN E. R. (1967). Regulation of glutamine synthetase VIII. ATP : glutamine synthetase adenylyltransferase, an enzyme that catalyses alterations in the regulatory properties of glutamine synthetase. Proc. Nat. Acad. Sci. U.S. 58, 1703-1715.

KINGDON H. S., HUBBARD J. S. and STADTMAN E. R. (1968). Regulation of glutamine synthetase. XI. The nature and implications of a lag phase in the Eschericia coli glutamine synthetase reaction. Biochemistry 7, 2136-2142.

KINGDON H. S. and STADTMAN E. R. (1967a). Two Escherichia coli glutamine synthetase with different sensitivities to feedback effectors. Biochem. Biophys. Research Comm. 27, 470-473.

KIRK P. L. (1950). Kjeldahl method for total nitrogen. Analyt. Chem. 22, 354-358.

KIRK P. R. and LEECH R. M. (1972). Amino acid biosynthesis by isolated chloroplasts during photosynthesis. Plant. Physiol. 50, 228-234.

KOHLAW G., DRAGERT W. and HOLZER H. (1965). Parallel-repression der Synthese von Glutamin-Synthetase und DPN-abhangiger Glutamatdehydrogenase in Hefe. Biochem. Z. 341, 224-228.

KOSTER A. L. (1963). Changes in the metabolism of isolated root systems of soy bean. Nature 198, 709-714.

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.

KRAMER J. (1970). NAD- and NADP-dependent glutamate dehydrogenase in Hydrogenomonas H 16. Arch. Mikrobiol. 71, 226-234.

LEA P. J. and FOWDEN L. (1975a). Asparagine metabolism in higher plants. Biochem. Physiol. Pflanzen 168, 3-14.

LEA P. J. and FOWDEN L. (1975b). The purification and properties of glutamine-dependent asparagine synthetase isolated from Lupinus albus. Proc. Roy. Soc., B. 192, 13-26.

LEA P. J. and MIFLIN B. J. (1974). Alternative route for nitrogen assimilation in higher plants. Nature 251, 614-616.

LEA P. J. and MIFLIN B. J. (1975). The occurence of glutamate synthase in algae. Biochem. Biophys. Res. Commun. 64, 856-862.

LEA P. J. and MIFLIN B. J. (1975). Glutamate synthase in blue-green algae. Biochem. Soc. Trans. 3, 381-384.

LEA P. J. and NORRIS R. D. (1976). The use of amino acid analogues in studies on plant metabolism. Phytochemistry 15, 585-595.

LEA P. J. and THURUMAN D. A. (1972). Intracellular location and properties of plant L-glutamate dehydrogenase. J. Exp. Bot. 23, 440-449.

LEAF G., GARDNER I.C. and BOND G. (1959). Observations on the composition and metabolism of the nitrogen-fixing root nodules of Myrica. Biochem. J. 72, 662-667.

LEECH R. M. and KIRK P. R. (1968). An NADP-dependent L-glutamate dehydrogenase from chloroplasts of Vicia faba L. Biochem. Biophys. Res. Commun. 32, 685-690.

LEES E. M., FARNDEN K. J. F. and ELLIOTT W. H. (1968). Studies in asparagine synthesis and utilization in seedlings. Arch. Biochem. Biophys. 126, 539-546.

LEWIS O.A.M. (1975). An ^{15}N-^{14}C study of the role of the leaf in the nitrogen nutrition of the seed of Datura stramonium L. J. Exp. Bot. 26, 361-366.

LEWIS O.A.M. and BERRY M. J. (1975). Glutamine as a major acceptor of reduced nitrogen in leaves. Planta (Berl.) 125, 77-80.

LEWIS O.A.M. and PATE J.S. (1973). The significance of transpirationally derived nitrogen in protein synthesis in fruiting plants of pea (Pisum sativum L.). J. Exp. Bot. 24, 596-606.

LE JOHN H. B. (1968). Unidirectional inhibition of glutamate dehydrogenase by metabolites. J. Biol. Chem. 243, 5126-5131.

LE JOHN H. B. (1971). Enzyme regulation, lysine pathways and all wall structures as indicators of major lines of evolution in fungi. Nature 231, 164-168.

LE JOHN H. B. and McCREA B. E. (1968). Evidence for two species of glutamate dehydrogenases in Thiobacillus novellus. J. Bacteriol. 95, 87-94.

LE JOHN H. B. and STEVENSON R. M. (1970). Multiple regulatory processes in nicotinamide adenine dinucleotide specific glutamic dehydrogenases. Biol. Chem. 245, 3890-3900.

LE JOHN H. B., SUSUKI I. and WRIGHT J. A. (1968). Glutamate dehydrogenase of Thiobacillus novellus. Kinetic properties and a possible control mechanism. J. Biol. Chem. 243, 118-128.

LEVER M. and BUTLER G. W. (1971). Precursors of asparagine in lupins. J. Exp. Bot. 22, 279-284.

LIGNOWSKI E. M., SPLITTSTOESSER W. E. and CHOU K. (1971). Glutamine synthesis in germinating seeds of Cucurbita moschata. Plant Cell Physiol. 12, 733-738.

LORENZ H. (1976). Nitrate, ammonium and amino acids in the bleeding sap of tomato plants in relation to form and concentration of nitrogen in the medium. Plant and Soil 45, 169-175.

MAGALHAES A. C., NEYRA C. A. and HAGEMAN R. H. (1974). Nitrogen assimilation and amino nitrogen synthesis in isolated spinach chloroplasts. Plant Physiol. 53, 411-415.

MARACHAL J., GUIZ C., GALVAING J. and GADAL P. (1977). Etude comparee des activites de la glutamate deshydrogenase, de la glutamine synthetase et de la glutamate synthase dans les racines et les feuilles de Phaseolus vulgaris L. et de Oryza sativa L. C.R. Acad. Sc. Paris, t. 284, 2111-2114.

MATSUMOTO H., WAKJUCHI N. and TAKAHASHI E. (1971). Some changes of some mitochondrial enzyme activities of cucumber leaves during ammonium toxicity. Physiol. Plantarum. 25, 353-357.

MEERS J. L. and TEMPEST D. W. (1970). Regulation of glutamine synthetase synthesis in some gram-negative bacteria. Biochem. J. 119, 603-605.

MEERS J. L., TEMPEST D. W. and BROWN C. M. (1970). Glutamine (amide) : 2-oxoglutarate, amino transferase oxido-reductase (NADP), an enzyme involved in the synthesis of glutamate by some bacteria. J. Gen. Microbiol. 64, 187-194.

MEERS J. L. and KJAERGAARD PEDERSEN L. (1972). Nitrogen assimilation by Bacillus licheniformis organisms growing in chemostat cultures. J. Gen. Microbiol. 70, 277-286.

MECKE D. and HOLZER H. (1966). Repression und Inaktivierung von Glutamin-Synthetase in Escherichia coli durch NH_4^+. Biochim. Biophys. Acta. 122, 341-351.

MEISTER A. (1962). Amino group transfer, p. 193-217. In. P. D. Boyer, H. Lardy and K. Myrbach (ed) The Enzymes, vol. 6. Acad. Press, Inc. New York.

McKENZIE H. A. and WALLACE H. S. (1954). The Kjeldahl determination of nitrogen. A critical study of digestion conditions - temperature catalyst and oxidizing agent. Aus. J. Chem. 7, 55-59.

McPARLAND R. H., GUEVARA J. G., BECKER R. R. and EVANS H. J. (1976). The purification and properties of the glutamine synthetase from the cytosol of soybean root nodules. Biochem. J. 153, 597-606.

MIFLIN B. J. (1970). Studies on the subcellular location of particulate nitrate and nitrite reductase, glutamic dehydrogenase and other enzymes in barley roots. Planta 93, 160-170.

MIFLIN B. J. (1974a). The location of nitrate reductase and other enzymes related to amino acid biosynthesis in the plastids of root and leaves. Plant Physiol. 50, 228-234.

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

MIFLIN B. J. and LEA P. J. (1975). Glutamine and asparagine as nitrogen donors for reductant-dependent glutamate synthesis in pea roots. Biochem. J. 149, 403-9.

MIFLIN B. J. and LEA P. J. (1976a). The pathway of nitrogen assimilation in plants. Phytochemistry 15, 873-885.

MIFLIN B. J. and LEA P. J. (1976b). The path of ammonia assimilation in the plant kingdom. Trends Biochem. Sci. 1, 103-106.

MIFLIN B. J. and LEA P. J. (1977). Amino acid metabolism. Ann. Rev. Plant. Physiol. 28, 299-329.

MILLER R. E. (1974). Glutamate synthase from Escherichia coli - an iron-sulfur flavoprotein. Separation and analysis of non-identical subunits. Biochem. Biophys. Acta. 364, 243.

MILLER R. E. and STADTMAN E. R. (1973). Glutamate synthase from Escherichia coli. An iron-sulfide flavoprotein. J. Biol. Chem. 247, 7407-7419.

MITCHELL D. J. and BIDWELL R. G. S. (1970). Compartments of organic acids in the synthesis of asparagine and homoserine in pea roots. Can. J. Bot. 48, 2001-2007.

MITCHELL C. A. and STOCKING C. R. (1975). Kinetics and energetics of light driven chloroplast glutamine synthetase. Plant. Physiol. Wash. 55, 59-63.

MONDER C. and JACOBSON B. (1964). Metal ion activators of glutamine synthetase. Biochem. Biophys. Res. Comm. 17, 136-140.

MORRIS I. and SYRETT P. J. (1965). The effect of nitrogen starvation on the activity of nitrate reductase and other enzymes in Chlorella. J. Gen. Microbiol. 38, 21-29.

NAGATINI H., SHIMUZU M. and VALENTINE R. C. (1971). The mechanism of ammonia assimilation in nitrogen fixing bacteria. Archiv fur Mikrobiologie 79, 164-175.

NICKLISH A., GESKE W. and KOHL J. G. (1976). Relevance of glutamate synthase and glutamate dehydrogenase to nitrogen assimilation of primary leaves of wheat. (Ger.). Biochem. Physiol. Pflanz. 170, 85-90.

NICLISCH A., TSENOVA E. N. and HOFFMAN P. (1977). Enzymological investigations on regulative interactions between N- and C-metabolism during illumination of etiolated primary leaves of Triticum aestivum L. (Ger.). Biochem. Physiol. Pflanzen 171, s. 375-384.

NIEMAN E. J. (1972). A study of translocation patterns of ^{14}C-labelled amino acids and amides in selected plant species. Ph.D. Thesis. University of South Africa.

OAKS A. (1967). Asparagine synthesis in Zea mays. Biochim. Acta. 141, 436-439.

OAKS A. and JOHNSON F. J. (1972). Cyanide as an asparagine precursor in corn roots. Phytochemistry 11, 3465-3471.

OREBAMJO T. O. and STEWART G. R. (1975). Some characteristics of nitrate reductase induction in Lemna minor. Planta (Berl.) 117, 1-10.

O'NEAL D. and JOY K. W. (1973a). Glutamine synthetase of pea leaves. 1. Purification, stabilization and pH optima. Arch. Biochem. Biophys. 159, 113-122.

O'NEAL D. and JOY K. W. (1973b). Localization of glutamine synthetase in chloroplasts. Nature New Biol. 246, 61-62.

O'NEAL D. and JOY K. W. (1974). Glutamine synthetase of pea leaves: Divalent cation effects, substrate specificity, and other properties. Plant Physiol. 54, 773-779.

O'NEAL D. and JOY K. W. (1975). Pea leaf glutamine synthetase. Regulatory properties. Plant Physiol. 55, 968-974.

OWNBY J. D. (1977). Effects of amino acids on methionine-sulfoximine induced heterocyst formation in Anabaena. Planta. 136, 277-279.

PAHLICH E. and HOFFMAN J. (1975). On the mechanism of action of glutamate dehydrogenase from pea seedlings and the regulation of the activity by adenosine phosphates, the energy charge and ions. Planta (Berl.) 122, 185-189.

PAHLICH E. and JOY K. W. (1971). Glutamate dehydrogenase from pea roots: Purification and properties of the enzyme. Can. J. Biochem. 49, 129-138.

PATE J. S. (1962). Root exhudation studies on the exchange of ^{14}C-labelled organic substances between roots and shoots of the nodulated legume. Plant and Soil 17, 333-347.

PATE J. S. and WALLACE W. (1964). Movement of assimilated nitrogen from the root system of the field pea (Pisum arvense L.). Ann. Bot. 28, 83-99.

PATE J. S. (1973). Uptake, assimilation and transport of nitrogen compounds by plants. Soil Biol. Biochem. 5, 109-119.

PATEMAN J. A. (1969). Regulation of synthesis of glutamate dehydrogenase and glutamine synthetase in micro-organisms. Biochem. J. 115, 769-775.

PRUSINER S. (1973). Glutaminases of Escherichia coli: properties, regulation and evolution. In: The Enzymes of Glutamine Metabolism. Ed. S. Prusiner and E. R. Stadman. Academic Press, New York and London.

PRUSINER S. and STADTMAN E. R. (1971). On the regulation of glutaminase in E. coli : metabolite control. Biochem. Biophys. Res. Comm. 45, 1474-1481.

RATHNAM C. K. M. and EDWARDS G. E. (1976). Distribution of nitrate assimilating enzymes between mesophyll protoplasts and bundle sheath cells in leaves of three groups of C_4 plants. Plant. Physiol. 57, 881-885.

RAVEL J. M., HYMPHREYS J. S. and SHIVE W. (1966). Control of glutamine synthesis in Lactobacillus arabinosus. Arch. Biochem. Biophys. 111, 720-727.

REHFIELD D. W. and TOLBERT N. E. (1972). Aminotransferases in peroxisomes from spinach leaves. J. Biol. Chem. 247, 4803-4811.

RHODES D., RENDON G. A. and STEWART G. R. (1975). The control of glutamine synthetase level in Lemna minor L. Planta (Berl.). 125, 201-211.

RHODES D., RENDON G. A. and STEWART G. R. (1976). The regulation of ammonia assimilating enzymes in Lemna minor. Planta (Berl.). 129, 203-210.

RHODES D. and STEWART G. R. (1974). A procedure for the in vivo determination of enzyme activity in higher plant tissue. Planta (Berl.). 118, 133-144.

RHODES D. D. and STEWART G. R. (1977). Glutamine synthetase and the control of nitrogen assimilation in Lemna minor L. Second Long Ashton Symposium. In Press.

RITENOUR G. L., JOY K. W., BUNNING J. and HAGEMAN R. H. (1967). Intracellular localization of nitrate reductase, nitrite reductase and glutamate dehydrogenase in green leaf tissue. Plant Physiol. 42, 233-237.

ROBERTSON J. G., WARBURTON M. P. and FARNDEN J. F. (1975). Induction of glutamate synthase during nodule development in lupin. FEBS Lett. 55, 33-37.

ROBINSON J. A. and STOCKING C. R. (1969). Oxygen evolution and the permeability of the outer envelop of isolated whole chloroplasts. Plant Physiol. 43, 1597-1604.

ROGNES H. (1975). A glutamine dependent asparagine synthetase from Lupinus luteus. Phytochemistry 14, 1975-1982.

RONZIO R. A., ROWE W. B. and MEISTER A. (1969). Studies on the mechanism of inhibition of glutamine synthetase by methionine sulfoximine. Biochemistry 8, 1066-1075.

ROWELL P., ENTICOTT S. and STEWART W. D. P. (1977). Glutamine synthetase and nitrogenase activity in the blue green alga Anabaena cylindrica. New. Phytol. 79, 41-54.

SAIGUSA M., OHIRA K. and FUJIWARA A. (1970). L. Glutamate dehydrogenase in higher plants. I Coenzyme specificity and intracellular localization. J. Sci. Soil and Manure (Japan) 41, 461-466.

SANTARIUS K. A. and STOCKING C. R. (1969). Intracellular localization of enzymes in leaves and chloroplast membrane permeability to compounds involved in amino acid synthesis. Z. Naturforsch. 246, 1170-1179.

SANWAL B. D. and LATA M. (1961). The occurence of two different glutamic dehydrogenases in Neurospora. Can. J. Microbiol. 7, 319-328.

SANWAL B. D. and LATA M. (1962). Concurrent regulation of glutamic acid dehydrogenases of Neurospora. Archs. Biochem. 97, 582-588.

SEGAL A., BROWN M. S. and STADTMAN E. R. (1974). Metabolite regulation of the state of adenylation of glutamine synthetase. Arch. Biochem. Biophys. 161, 319-327.

SHANER D. L. and BOYER J. S. (1976). Nitrate reductase activity in maize (Zea mays L.) leaves. I. Regulation by nitrate flux. Plant Physiol. 58, 499-504.

SHAPIRO B. M. (1969). The glutamine synthetase deadenylating system from Escherichia coli, resolution into two components, specific nucleotide stimulation, and cofactor requirements. Biochemistry 8, 659-670.

SHAPIRO B. M. and GINSBURG A. (1968). Effects of specific divalent cations on some physical and chemical properties of glutamine synthetase from Escherichia coli. Taut and relaxed enzyme forms. Biochem. 7, 2153-2167.

SHAPIRO B. M. and STADTMAN E. R. (1967). Regulation of glutamine synthetase IX. Reactivity of the sulphydryl groups of the enzyme from Escherichia coli. J. Biol. Chem. 242, 5069-5079.

SHAPIRO B. M. and STADTMAN E. R. (1968). 5-adenylyl-O-tyrosine, the novel phosphodiester residue of adenylated glutamine synthetase from Escherichia coli. J. Biol. Chem. 243, 3769-3771.

SHAPIRO B. M. and STADTMAN E. R. (1970). The regulation of glutamine synthesis in micro-organisms. Ann. Rev. Microbiol. 24, 501-518.

SHEPARD D. V. and THURUMAN D. A. (1973). Effects of nitrogen sources on the activity of L-glutamate dehydrogenase of Lemna minor. Phytochemistry 12, 1937-1946.

SIMONIS W. and URBACH W. (1971). Photophosphorylation in vivo. Ann. Rev. Plant Physiol. 24, 89-114.

SIMS A. P. (1976). Regulation of glutamine metabolism in fungi with particular reference to the food yeast Candida utilis. Pers. Exp. Biol. 2, 247-261.

SIMS A. P. and FOLKES B. F. (1964). A kinetic study of the assimilation of ^{15}N-ammonia and the synthesis of amino acids in an exponentially growing culture of Candida utilis. Proc. Roy. Soc. B. 159, 479-484.

SIMS A. P., FOLKES B. F. and BUSSEY A. H. (1968). Mechanisms involved in the regulation of nitrogen assimilation in micro-organisms and plants in Recent Aspects of Nitrogen Metabolism in Plants, First Long Ashton Symposium, 1967, ed. Hewitt, E. J. and Cutting, C. V. pp. 91-114. Academic Press, London and New York.

SIMS A. P., TOONE J. and BOX V. (1974). The regulation of glutamine metabolism in Candida utilis: Mechanisms of control of glutamine synthetase. J. Gen. Microbiol. 84, 149-162.

STEWART G. R. (1972). The regulation of nitrite reductase level in Lemna minor L. J. Exp. Bot. 23, 171-183.

STEWART G. R. and RHODES D. (1976). Evidence for assimilation of ammonia via the glutamine pathway in nitrate grown Lemna minor L. FEBS Lett. 46, 340-342.

STEWART G. R. and RHODES D. (1977). A comparison of the characteristics of glutamine synthetase and glutamate dehydrogenase from Lemna minor L. New Phytol. 79, 257-268.

STEWART W. D. P. and ROWELL P. (1975). Effects of L-methionine-DL-sulfoximine on the assimilation of newly fixed NH_3, acetylene reduction and heterocyst production in Anabaena cylindrica. Biochem. Biophys. Res. Comm. 65, 846-856.

STREETER J. G. (1973). In vivo and in vitro studies on asparagine biosynthesis in soybean seedlings. Arch. Biochem. Biophys. 157, 613-624.

TATE S. S. and MEISTER A. (1971). Regulation of rat liver glutamine synthetase: Activation by a-ketoglutarate and inhibition by glycine, alanine and carbonyl phosphate. Proc. Nat. Acad. Sci. U.S. 68, 781-785.

TEMPEST D. W., MEER J. L. and BROWN C. M. (1970). Synthesis of glutamate in Aerobacter aerogenes by a hitherto unknown route. Biochem. J. 117, 405-407.

THOMAS J., WOLK C. P., SCHAFFER P. W., AUSTIN S. M. and GALONSKY A. (1975). The initial organic products of fixation of ^{13}N-labelled nitrogen gas by the blue green alga Anabaena cylindrica. Biochem. Biophys. Res. Comm. 67, 501-507.

THURUMAN D. A., PALIN C. and LAYCOCK M. V. (1965). Isoenzymatic nature of L-glutamic dehydrogenase of higher plants. Nature 207, 193-194.

TOLBERT N. E. (1971). Microbodies - peroxisomes and glyoxysomes. Ann. Rev. Plant Physiol. 22, 45-74.

TOLBERT N. E. (1973). Compartmentation and control in microbodies. Symp. Soc. Exp. Biol. 27, 215-239.

TROMP J. and OVAA J. C. (1976). Effect of time of nitrogen application on amino-nitrogen composition of roots and xylem sap of apple. Physiol. Plant. 37, 29-34.

TSENOVA E. N. (1972). Isolation and properties of glutamate dehydrogenase from pea chloroplasts. Enzymologia 43, 397-408.

TSUKAMOTO A. (1970). Reductive carboxylation and amination of keto acids by spinach chloroplasts. Plant Cell Physiol 11, 221-230.

TYCHSEN K. (1976). Irradiance and nitrogen metabolism in spinach. Acta Agric. Scandinavica 26, 189-195.

UMBARGER H. E. (1969). Regulation of amino acid metabolism. Ann. Rev. Biochem. 38, 323

VALENTINE R. S., SHAPIRO B. M. and STADTMAN E. R. (1968). Regulation of glutamine synthetase. XII Electron microscopy of the enzyme from Escherichia coli. Biochemistry 7, 2143-2152.

VARNER J. E. (1960). The optical specificity of glutamine synthetase. Arch. Biochem. Biophys. 90, 7-11.

VARNER J. E. and WEBSTER G. (1955). Studies on the enzymatic synthesis of glutamine. Plant Physiol. 30, 393-402.

VENDER J., JAYARAMAN K., RICKENBERG H. (1965). Metabolism of glutamic acid in a mutant of Escherichia coli. J. Bacteriol 90, 1304-1307.

VERNON L. P., SHAW E. R. and KE B. (1966). Photochemically active particle derived from chloroplasts by the action of the detergent Triton X-100. J. Biol. Chem. 241, 4101-4109.

WALDRON J. C. (1976). Nitrogen compounds transported in the xylem of sugar cane. Aust. J. Plant Physiol. 3, 415-9.

WALLACE W. and PATE J. S. (1967). Nitrate assimilation in higher plants with special reference to the cocklebur (Xanthium pennsylvanicum Walr.) Ann. Botany 31, 213-228.

WALLSGROVE R. M., HAREL E., LEA P. J. and MIFLIN B. J. (1977). Studies on glutamate synthase from the leaves of higher plants. J. Exp. Bot. 28, 588-596.

WEBSTER G. C. and VARNER J. E. (1955). Aspartate metabolism and asparagine synthesis in plant systems. J. Biol. Chem. 215, 91-99.

WEISSMAN G. S. (1964). Effect of ammonium and nitrate nutrition on protein level and exhudate composition. Plant Physiol. 39, 947-952.

WEISSMAN G. S. (1972). Influence of ammonium and nitrate nutrition on the pyridine and adenine nucleotides of soybean and sunflower. Plant Physiol. 49, 142-145.

WELANDER M. (1974). Enzyme activities in Urtica dioica: Effect of daylength and leaf age on glutamate dehydrogenase, aspartate aminotransferase and alanine aminotransferase. Physiol. Plant 30, 192-199.

WESTPHAL H. and HOLZER H. (1964). Synthese von NAD-abhangiger glutamat-dehydrogenase in protoplasten von Saccharomyces calsbergensis. Biochim. Biophys. Acta. 89, 42-46.

WOLK C. P., THOMAS J. and SHAFFER P. W. (1976). Pathway of nitrogen metabolism after fixation of ^{13}N-labelled nitrogen gas by the cyanobacterium, Anabaena cylindrica. J. Biol. Chem. 251, 5027-5034.

WOOLFOLK C. A., SHAPIRO B. M. and STADTMAN E. R. (1966). Regulation of glutamine synthetase I. Purification and properties of glutamine synthetase from Escherichia coli. Arch. Biochem. Biophys. 116, 177-192.

WOOLFOLK C. A. and STADTMAN E. R. (1967). Regulation of glutamine synthetase III Cumulative feedback inhibition of glutamine synthetase from E. coli. Arch. Biochem. Biophys. 118, 736-755.

WU G. and YUAN L. H. (1968). Regulation of synthesis of glutamine synthetase in Escherichia coli. J. Gen. Microbiol. 51, 57-66.

YEM E. W. and WILLIS A. J. (1956). The respiration of barley plants IX. The metabolism of roots during the assimilation of nitrogen. New Phytol. 55, 229-238.

YONEYAMA T., AKIYAMA Y. and KUMAZAWA K. (1977). Nitrogen uptake and assimilation by corn roots. Soil Sci. Plant Nutr. 23, 85-91.

YONEYAMA T., KOMAMURA K. and KUMAZAWA K. (1975). Nitrogen transport in intact corn roots. Soil Sci. Plant Nutr. 12, 371-377.

YONEYAMA T. and KUMAZAWA K. (1974). A kinetic study of the assimilation of ^{15}N-labelled ammonium in rice seedling roots. Plant Cell Physiol. 15, 655-661.

YONEYAMA T. and KUMAZAWA K. (1975). A kinetic study of the assimilation of ^{15}N-labelled nitrate in rice seedlings. Plant Cell Physiol. 16, 21-26.

YUE S. B. (1969). Isoenzymes of glutamate dehydrogenase in plants. Plant Physiol. 44, 453-457.

ZELITCH I. (1972). Comparison of the effectiveness of glycolic acid and glycine substrates for photorespiration. Plant Physiol. 50, 109-113.