# AN INVESTIGATION OF THE PATHWAY OF NITROGEN INCORPORATION INTO THE LEAF AND ROOT METABOLISM OF *HELIANTHUS ANNUUS* L.

by

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CONTENTS

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## ABBREVIATIONS AND SYMBOLS

| | |
|---|---|
| ALA | alanine |
| ARG | arginine |
| ASG | 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.

Xylem sap from hydroponically-grown and vermiculite-grown Helianthus annuus fed at 50 $\mu$g N $cm^{-3}$ and 300 $\mu$g N $cm^{-3}$ feeding level was analysed for nitrate, ammonia and amino compounds. Nitrate accounted for 77% to 94% of the total nitrogen transported via the xylem stream.

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

Excised leaves received feeding solutions at two concentrational levels: 300 $\mu$g N $cm^{-3}$ and 50 $\mu$g N $cm^{-3}$ via the xylem stream prior to experimentation. Nitrate - $^{15}N$ xylem stream and infiltration feeding experiments on Helianthus leaves indicated an apparent major routing of newly reduced nitrogen to glutamine. Of the other major soluble amino compounds, alanine, aspartate, serine and glycine were found to be important in the primary assimilation of newly reduced nitrogen.

After pretreatment with feeding solutions containing 5mM methionine sulphoximine, and after infiltration with $^{15}N$-nitrate containing 5mM methionine sulphoximine, complete supression of nitrogen routing into amino compounds was noted with the resultant accumulation of $^{15}N$ in a large ammonia pool.

Conditions of nitrogen stress were indicated from the concentrational changes of free amino compound pools after methionine sulphoximine treatment. Glutamate dehydrogenase activity (NADH-dependent) was not inhibited by the methionine sulphoximine pretreatment.

Assimilation of newly reduced nitrogen into amino compounds in Helianthus leaves via the glutamine synthetase/ glutamate synthase pathway, irrespective of nitrogen availability, was indicated by these results. There was a decrease in the $^{15}N$ incorporation at the higher feeding level caused possibly, by a decrease in glutamate synthase activity.

The incorporation of $^{15}N$-nitrate into the free amino compounds of Helianthus roots indicated that they are capable of reduced nitrate-N assimilation, although at a much lower rate than in the leaves. Glutamine was the most heavily $^{15}N$-labelled soluble amino compound at both feeding levels used. The effect of feeding Helianthus roots with $^{15}N$-nitrate solutions containing 7mM 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 nitrogen incorporation into leaf and root metabolism of *Helianthus annuus* L. using $^{15}N$ tracer feeding experiments (with and without enzyme inhibition), and enzymological assays. High and low nitrogen feeding levels, were used to determine whether nitrogen concentration could affect the pathway of ammonia assimilation. The feeding of nitrate nitrogen was preferred to ammonia nitrogen for various reasons:

(a) Ammonia is not normally a major constituent of the xylem sap providing the leaf with its nitrogen supply;

(b) Nitrate is the most readily available form of nitrogen in most soils, (Hewitt *et al*, 1976);

(c) The nitrate/nitrite reduction stages of nitrate incorporation may constitute a regulatory activity important in the normal physiological behaviour of a plant.

# CHAPTER 2

## INTRODUCTION

Nitrogen constitutes approximately 2 percent and carbon approximately 40 percent of the dry weight of plants on a world wide scale (Delwiche, 1977). It is estimated that 200 billion tons of carbon are fixed annually by photosynthetic processes (Galston, 1961) which on a basis of approximate analysis would require the incorporation of 10 billion tons of nitrogen. Although nitrogen constitutes a comparately small fraction of the elements in the earth's crust, (less than 0,005 percent by weight exclusive of that contained in the atmosphere and the ocean), it's comparative abundance in the atmosphere (78.1 percent on a molar basis), and its ubiquitous and important role in the biosphere make its metabolism of extreme interest to the plant biologist (Delwiche, 1977).

Apart from those species that have a symbiotic association with nitrogen-fixing bacteria, the bulk of plant nitrogen arises from the reduction of nitrate taken up from the soil (Beevers and Hageman, 1969). Plants utilize ammonia and/or nitrate as nutrients. These inorganic forms of nitrogen are assimilated and go to make up the amino acids and proteins in plants. Since reduction of nitrate to the usable (ammonium) form requires the donation of 8 electrons, while carbon requires but 4 electrons, the reduction of nitrate to ammonia utilises a massive amount of solar energy. Only nitrogen in the fully reduced state, can be assimilated into

organic compounds. Reduction of nitrate to nitrite and then to ammonia by plant enzymes was demonstrated by Hageman, Cresswell and Hewitt (1962). These enzymes (nitrate reductase E.C. 1.6.6.1 and nitrite reductase E.C. 1.6.6.4) are now well documented (See Reviews by Beevers and Hageman, 1969; Hewitt et al, 1976). The mechanism of ammonia assimilation into organic nitrogen, however, is less clearly understood and is discussed in the remainder of this Chapter.

## 2.1 Glutamate Dehydrogenase

The experiments by Cocking and Yemm (1961) using $^{15}NH_4^+$ with young barley seedlings showed that there was a high initial incorporation of $^{15}N$ into glutamate and glutamine. From further work, including the careful kinetic studies of ammonia -$^{15}N$ incorporation in intact Chlorella cells by Bassham and Kirk (1964) and the yeast Candida utilis by Sims and Folkes (1964), it has been assumed that the synthesis of glutamate by the reductive amination of 2-oxoglutarate, catalysed by glutamate dehydrogenase (L-glutamate: $NAD^+$ oxidoreductase, deaminating E.C. 1.4.1.2) (GDH) is the mechanism of primary ammonia assimilation in plant cells, Reaction (1).

(1) 2-oxoglutarate + $NH_4^+$ + NAD(P)H $\rightleftharpoons$ L-glutamate + $H_2O$ + $NAD(P)^+$

The reaction has been shown to be reversible (Ehmke and Hartman, 1978) and can constitute an important catabolic process (Bryan, 1976). Purification and characterization have been well documented for animal GDH (Goldin and Frieden,

1971) but not so well for plants. Work on purification and characterization has been carried out in a few instances (Barratt and Strickland, 1963; Pahlich and Joy, 1971; Davies and Teixeira, 1975; Garland and Dennis, 1977; Fawole and Boulter, 1977; Lea and Thurman, 1972). The molecular weight for the enzyme varies, depending on whether it is chloroplastic or mitochondrial in origin, and if it was determined for NAD and NADP separately or not. The molecular weight for pea root GDH is 208 000 (Pahlich and Joy, 1971) and that of pea seedling mitochondrial GDH 230 000 (Davies and Teixeira, 1975); 250 000 for the bacteria Mycoplasma laidlawii GDH (Brown et al, 1974). There appears to be disagreement in the figures for Neurospora crassa. Barratt and Strickland, (1963) found the GDH molecular weight to be 267 400 ($\pm$3 000) whereas Grover and Kapoor, (1973) found the molecular weight to be 320 000. Lea and Thurman, (1972) did rather extensive work on Lactuca sativa GDH reporting the following molecular weights: NAD mitochondrial 259 000, NADP mitochondrial 267 000, NAD chloroplastic 304 000, and NADP chloroplastic 311 000.

The GDH in higher plants was thought to be mainly located in the mitochondria and to be NAD-specific (Ritenour et al, 1967; Gayler and Morgan, 1976). Work by Leech and Kirk on Vicia faba, (1968) indicated that chloroplasts also possess the enzyme and that it is NADP-specific, whereas others found the chloroplastic enzyme to be NAD-specific (Santarius and Stocking, 1969). Sims, Folkes and Bussey

working with Candida utilis, (1968) made claims for the presence of a NAD-specific GDH in leaves and a NADP-specific enzyme in roots, and they failed to detect any NADP-dependent activity in leaves. Grimes and Fottrell, (1966) claimed to have separated NAD- from NADP-dependent GDH activities in root nodules of Trifolium repens. Harel, Lea and Miflin, (1977) indicate that the location of NADH-specific GDH appears to be exclusively in the bundle sheath and NADPH-specific GDH in both bundle sheath and mesophyll of maize leaves. Joy, (1969) suggested that, as a result of detecting a different effect of $NH_4^+$ on the activity of NAD- and NADP-dependent GDH in extracts of Lemna minor, that two different enzymes were responsible for the observed activities. There is evidence to support this idea, (Sims, Folkes and Bussey, 1968; Sanwal and Lata, 1961) whilst other workers propose a single enzyme lacking specificity for the coenzyme, (Bone, 1959; Yue, 1969; Pahlich and Joy, 1971; Ehmke and Hartman, 1976; Fawole and Boulter, 1977). Although there are claims for the existence of separate NAD-dependent GDH and NADP-dependent GDH, located in different parts of the same plant (Grimes and Fottrell, 1966; Leech and Kirk, 1968; Sims et al, 1968; Lea and Thurman, 1972), there has not been a case where two distinct GDH proteins have been separated from higher plants as has been done with fungi (Sanwal and Lata, 1961; Dennen and Niederpruem, 1967) and some bacteria (Lé John and McCrea, 1968; Kramer, 1970; Joseph and Weixom, 1970). Weissman, (1972b) showed the

influence of ammonia and nitrate nutrition on the pyridine nucleotides of soybean and sunflower. Total pyridine nucleotide concentration of root tissue was the same with either nutrient, but ammonia nutrition yielded a high concentration of NAD, while nitrate showed high NADP activity. It was proposed, using the two-enzyme system (Joy, 1969), that the NAD-linked enzyme fulfilled a catabolic role while the NADP-linked enzyme was anabolic (Sanwal and Lata, 1961, 1962). With dual coenzyme specificity and with single coenzyme specificity, GDHs exist that have anabolic or catabolic roles (Brown et al, 1974). In many organisms, the anabolic GDHs are repressed when environmental (or pool) ammonia has a low concentration and have high activity when pool ammonia is high (Brown et al, 1972). In others, however, one GDH may serve a dual role and be under complex control (Brown et al, 1974); and in yet others with both NAD- and NADP-linked enzymes, the former behaves as a catabolic enzyme relatively unresponsive to ammonia concentration, while the NADP enzyme is anabolic and repressed in low ammonia conditions (Brown et al, 1974).

The NAD-specific GDH has been shown to be allosteric (Lé John 1968) and is specifically regulated by AMP, which acts as an activator in the breakdown of glutamate and as an inhibitor in the formation of glutamate, suggesting that it (NAD-specific GDH) acts in a purely catabolic role in the cell. Lé John, Suzuki and Wright, working with Thiobacillus novellus (1968) proposed that the primary function of NAD-specific GDH is to generate NADH and in turn

ATP, with concomitant breakdown of glutamate. ATP utilizing reactions would provide the effector, AMP, which would continuously regulate the equilibrium in favour of glutamate catabolism. The anabolic role of NADP-specific GDH and the catabolic role of NAD-specific GDH have been shown for most bacteria and fungi (Brown *et al*, 1974). These regulatory properties of GDH are important in the study of the phylogenetic origins of the fungi, since more than forty species of lower fungi, Myxomycetes and Phycomycetes, were found to possess only an NAD-linked GDH while the higher fungi, Deuteromycetes, Ascomycetes and Basidiomycetes seem to produce both NAD and NADP-linked GDHs (Lé John, 1971).

Isoenzymes of GDH have been reported by various workers. Some of the results reported are 7 isoenzymes from albumins extracted from *Vicia faba* and *Pisum sativum* (Thurman *et al*, 1965), 3 isoenzymes from root nodules (Grimes and Fottrell, 1966) and a range of 7 for 3 day old to 4 for 26 day old mung bean hypocotyls (Yue, 1969). Yue, (1969) found that the isoenzymes had the same coenzyme specificity and were localized in the mitochondrial fraction of the cell in all plants examined. There is no evidence for separate isoenzymes (Lea and Thurman, 1972), one active only with NADH and one with NAD(P)H as is apparently the case in bacteria and fungi (Brown *et al*, 1974).

Fawole and Boulter, (1977) demonstrated that the GDH activity in *Vigna unguiculata* mitochondria was, with NADH,

$NAD^+$ and NADPH in the ratios of 126:2:2. Kanamori et al, (1972) also found a much greater activity with the (aminating) NADH and NADPH, than with the (deaminating) $NAD^+$ in rice roots. Davies and Teixeira, (1975) suggested that the ratio NADH:$NAD^+$ may play an important role in the control of glutamate metabolism. In a comparison of the purified mitochondrial and supernatant enzyme preparation from pea epicotyls, Davies and Teixeira, (1975) showed the ratio: $V_{max}$ NADH oxidation/$V_{max}$ $NAD^+$ reduction: was 25 for both preparations indicating that the mitochondrial preparation acts in an anabolic role catalysing the synthesis of glutamate. Working on highly purified (125-fold purification) pea root GDH, Pahlich and Joy, (1971) showed NADH (aminating) and $NAD^+$ (deaminating) activities, but the ratio of these activities was not constant and could be changed experimentally by the extraction buffer (tris or phosphate) or by the presence of $Ca^{2+}$, $Zn^{2+}$ or EDTA. Shepard and Thurman, (1973) and Joy, (1971) working with Lemna mitochondrial NAD-dependent GDH have shown that the enzyme may have an assimilatory role. However, the in vivo role of mitochondrial GDH still remains obscure.

Bassham and Kirk, (1964) indicated that reductive amination to form glutamate is the primary route for incorporation of $^{15}N$ in Chlorella pyrenoidosa and that this would be primarly in the chloroplasts. The location of nitrite reductase in the chloroplast (Betts and Hewitt, 1966; Ritenour, et al, 1967; Swader and Stocking, 1971; Dalling et al, 1972)

would seem to be the most logical site for amino acid biosynthesis. Magalhaes et al, (1974) have supported this hypothesis by showing that isolated spinach chloroplasts, reducing $NO_2^-$ and synthesizing amino-nitrogen *de novo*, are light dependent.

One difficulty in ascribing chloroplastic glutamate formation to the activity of chloroplastic GDH, in higher plants, is concerned with its Km for ammonia. Although Tsenova, (1972) found the Km from pea chloroplasts to be $4.24 \times 10^{-5}$M, other workers (Bulen, 1956; Lea and Thurman, 1972; Magalhaes et al, 1974; Davies and Teixeira, 1975) found values in excess of 2mM, the level at which ammonia uncouples chloroplasts (Good, 1960). The Km for ammonia for most bacterial GDH is $\pm$4mM indicating a low affinity of the enzyme for its substrate (Brown *et al*, 1972). In *Chlorella vulgaris*, the NADP-dependent GDH has a Km value of 10mM but the internal ammonia pool ranges from 5-10mM, independent of the nitrogen source. Similar results were found for the marine diatom *Ditylium brightwellii* (Eppley and Rogers, 1970). The alga *Caulerpa simpliciuscula* has been shown (Gayler and Morgan, 1976) to have an NADP-dependent GDH with a sufficiently low Km of 0,67mM for ammonia, which would provide a direct and economical route to glutamate. It is always possible that the *in vivo* Km of chloroplastic GDH is different from that of the isolated enzyme (Bahr and Jensen, 1974) or that the enzyme is in close physical proximity to nitrite reductase in a special compartment of the chloroplast (Miflin and Lea, 1976).

## 2.2 Glutamine Synthetase

Another reason (other than Km previously mentioned) four doubting the role of glutamatedehydrogenase in ammonia assimilation in chloroplasts is the finding by several groups that chloroplasts contain glutamine synthetase (Santarius and Stocking, 1969; O'Neal and Joy, 1973b; Haystead, 1973). Glutamine Synthetase (GS) (E.C.6.3.1.2), which catalyzes the synthesis of glutamine from glutamate, ammonia, and ATP (Reaction 2), provides another means for ammonia assimilation.

(2) Glutamate + $NH_4^+$ + ATP $\longrightarrow$ Glutamine + ADP + Pi + $H_2O$

O'Neal and Joy (1973b), using marker enzymes, with peas, suggest that at least 32% and probably more than 66% of higher plant GS is located in the chloroplast. There is no evidence for the presence of the enzyme in any other organelle, but it probably is present in the plastids (Miflin, 1974; Washitani and Sato, 1977).

The rates of photoreduction of 2-oxo glutarate by isolated chloroplasts are low, being 0,4 µmole (mg Chl)$^{-1}$h$^{-1}$ for spinach (Tsukamoto, 1970) and 0,6 µmole (mg Chl)$^{-1}$h$^{-1}$ for 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 (9,0 µmole (mg Chl)$^{-1}$h$^{-1}$ , Miflin, 1974 and 12,0 µmole

(mg Chl)$^{-1}$h$^{-1}$ , Magalhaes et al, 1974). GS activity (90 µmole (mg Chl)$^{-1}$h$^{-1}$) in spinach leaves, (Miflin, 1974) being several times that of GDH, is more than sufficient to cope with the reported rates of amino nitrogen production. GS has a low Km for ammonia: Km = 19 µM in pea leaves (O'Neal and Joy, 1974); Km = 0,3 mM in peas (Varner, 1960); Km = 0,67 mM in pumpkin seeds (Lignowski et al, 1971); quoted by O'Neal and Joy, 1974
Km = 0,4 mM in rice (Caldos, 1971^); and Km=15µm in Lemna minor (Stewart and Rhodes, 1977). Illuminated chloroplasts will convert glutamate to glutamine at a rate of approximately 10 µmoles (mg Chl)$^{-1}$h$^{-1}$ which is roughly comparable to the observed rates of nitrite reduction to ammonia (Miflin, 1974). Therefore, it is unlikely that GDH could compete successfully with GS for ammonia in the chloroplast.

The light dependence of the process suggests that ATP generated by photophosphorylation is used in the GS catalysed reaction. Givan (1975, 1976) working with peas, has suggested that ATP necessary to support chloroplastic GS may be derived from noncyclic or pseudocyclic phosphorylation (requiring photosystems I and II). Others have suggested that the operation of a cyclic photophosphorylation process, dependent entirely on photosystem I, could be operating, (Mitchell and Stocking, 1975; Simonis and Urbach, 1971). The light-driven GS absolutely requires either, or both forms of photosynthetically generated ATP, but there is no conclusive evidence to say which one is operating (Mitchell and Stocking, 1975; Givan, 1976).

GS has been purified from pea leaves by O'Neal and Joy (1973a, and 1974) and from soybean root nodules by McParland et al, (1976). The molecular weight was found to be in the range of 330 000 to 376 000 which is smaller than that found for E. coli (592 000) (Shapiro and Ginsburg, 1968). A two planar structure has been reported for E. coli GS (Valentine et al, 1968) and appears to be a common feature of all GSs (Miflin and Lea, 1977). One of the most unusual properties of the enzyme is the effect of divalent cation species, and their concentration, on the substrate specificity and kinetic behaviour of the enzyme. There are pronounced differences in pH optima, varying from 5.2 to 8.3, depending on the divalent cations used and their concentrations (O'Neal and Joy 1973a, 1974). The purified enzyme has an absolute requirement for a divalent cation such as $Mg^{2+}$, $Mn^{2+}$, and $Co^{2+}$, which are the three most effective cations (O'Neal and Joy, 1974). In pea leaves (O'Neal and Joy, 1975), $Mn^{2+}$ results in a low specificity for nucleotide triphosphate. This is in agreement with the results found for Bacillus subtilis (Deuel and Stadtman, 1970). The carrot enzyme was most active with $Mg^{2+}$ (Caldos, 1971 quoted by O'Neal and Joy, 1974) as is the pea leaf enzyme (O'Neal and Joy, 1974). Varner (1960) found GS to be most active with $Co^{2+}$ followed closely by $Mg^{2+}$ in pea seeds. The relative physiological significance of the activity of these divalent cations is difficult to establish, in the absence of precise information on the level of these ions at the site of GS

in the cell (Miflin and Lea, 1977).

## 2.3 Discovery of Glutamate Synthase

If glutamine synthetase is the route of entry for ammonia into plant amino acid metabolism, some enzyme or enzyme system must exist to transfer the amide group of glutamine to the $\alpha$-keto group of an amino acid. The pioneering work on such an enzyme was done by Tempest *et al*, (1970) working on the bacterium *Aerobacter aerogenes*. As has already been observed (Section 2.1) the Km for ammonia of most bacterial GDH is about 4 mM (Miflin and Lea, 1976) indicating a low affinity of the enzyme for its substrate. In cases where ammonia is limiting and its intracellular concentration is of the order of 0,5mM, Tempest *et al*, (1970) observed that ammonia could only be assimilated if production of GDH was greatly increased. However, when they measured the level of GDH in ammonia-limited chemostat cultures of *Klebsiella aerogenes* they found it to have dropped to 3% of its original level. This behaviour is inconsistent with GDH being responsible for ammonia assimilation. Tempest and coworkers thought that it was possible that a clue to the pathway of incorporation of ammonia into amino acids could be obtained by adding a pulse of ammonia to the culture (disturbing the steady state). A 25-fold increase in the free glutamine concentration was observed with an 80-fold increase in the glutamine synthetase activity after such an experiment was performed (Tempest *et al*, 1970). This rapid

rise in glutamine was followed by a secondary formation of glutamate (Tempest et al, 1970; Meers et al, 1970). Although the first part of the reaction can be carried out by GS no enzyme was yet known that could catalyze the transfer of the amide nitrogen of glutamine into the 2-amino position of glutamate.

Tempest, Meers and Brown (1970) working with several bacteria were able to demonstrate the existence of an enzyme which catalyzed the pyridine nucleotide-dependent reductive transfer of the amido group of glutamine to 2-oxoglutarate, resulting in the production of two molecules of glutamate (Reaction 3):

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

This reaction has been shown to be unidirectional (Nagatine et al, 1971; Brown et al, 1972). This enzyme was originally called glutamine (amido): 2-oxoglutarate amino-transferase (oxidoreductase NADP), trivial name glutamate synthase, and is often referred to by its acronym GOGAT. However, it has recently been reclassified by the International Union of Biochemistry (I.U.B.) as E.C. 1.4.1.13, systematic name L. Glutamate: $NADP^+$ oxidoreductase (transaminating).

The synthesis of glutamate from $NH_3$ and 2-oxoglutarate, via glutamine and GOGAT, requires the participation of an active GS. Significantly, bacterial GS is generally found in considerable amounts in organisms where growth has been limited by $NH_3$ deprivation (Woolfolk et al, 1966; Wu and

Yuan, 1968; Pateman, 1969).

The net result of the GDH and GS/GOGAT pathways is the same. The GS/GOGAT functions in low-ammonia environments, however, and involves the participation of ATP. Presumably this expenditure of energy, associated with the synthesis of glutamine, is the 'price that must be paid' by the bacteria in order to assimilate low intracellular concentrations of ammonia (Tempest *et al*, 1970). The ATP driven conversion of ammonia to an amide group could act as a 'pump' which could scavenge the last trace of ammonia from the environment (Umberger, 1969). Since this alternative pathway of glutamate synthesis was first reported (Tempest *et al*, 1970) it has been shown to operate in many other bacterial species (Meers *et al*, 1970; Dainty, 1972; Brown *et al*, 1972, 1974; Brooks and Meers, 1973). The presence of the GS/GOGAT system in symbiotic nitrogen-fixing associations has been reported (Nagatini *et al*, 1971; Kennedy, 1973; Dunn and Klucas, 1973; Ryan and Fottrell, 1974), but with GOGAT at levels probably only sufficient to cope with 10-20% of the nitrogen flux. Robertson et al, (1975) have found, considerable levels of GOGAT in *Lupinus* root tissue in association with nodule formation.

GOGAT has been purified from *E. coli* by Miller and Stadtman (1972) and Miller (1974). It is a flavoprotein containing nonheam iron and labile sulphur. The data suggest that the enzyme consists of eight subunits, four each of two types, with molecular weights of 135 000 and

53 000 aggregated to give a final MW of 800 000. Each complex probably contains 32 iron atoms, 32 labile sulphide atoms and 8 non-covalently linked flavine molecules. From spectroscopic measurements of the purified enzyme it is suggested that the nature of the iron-protein bonding might be similar to that found in E. coli (Miller and Stadtman, 1972). The reaction mechanism is thought to occur in two stages, the first involves the reduction of the enzyme-bound flavine with $NADPH_2$ or sodium dithionite as electron donor, and the second the reductive transfer of the glutamine amide nitrogen to 2-oxo-glutarate, forming two molecules of glutamate. The enzyme is highly specific for glutamine with Km values of 0,25mM (Miller and Stadtman, 1972); and 0,2mM (Dainty, 1972) and 2-oxoglutarate with Km values of 7,3 μM (Miller and Stadtman, 1972) and 0,2mM (Dainty, 1972).

Meers et al, (1970) and Meers and Kjaergaard-Pedersen (1972) found that the enzyme from a variety of organisms could not accept an alternative electron donor to $NADPH_2$. Other workers (Nagatini et al, 1971; Brown et al, 1972) found that some species of bacteria synthesized an enzyme that was specific for $NADH_2$. No organism has yet been isolated that produces separate $NADH_2$-linked enzymes, but Dainty (1972) has reported that the enzyme from Clostridium pasteurianum has dual coenzyme specificity.

Dougall (1974) working with carrot cell cultures, and Fowler et al, (1974), working with sycamore and pea

root cells, first reported GOGAT activity in higher plants which differed from the bacterial enzymes, in that it was active with both NADH and NADPH as electron donors. GOGAT from the pea cotyledon showed that both pyridine nucleotides serve as reductant in the enzyme reaction (Beevers and Storey, 1976). However, it has been suggested that NADPH is converted by a pyridine nucleotide phosphatase to NADH (Storey and Reporter, 1978; Beevers and Storey, 1976; Wells and Hageman, 1974) and that the requirement could actually be for NADH as is the case for lupin roots (Robertson et al, 1975). Dougall and Bloch (1976) suggested, using cell cultures of carrots, that two different enzymes are responsible for the GOGAT activity observed rather than a single enzyme which can utilize both reduced pyridine nucleotides.

Lewis and Pate (1973) in experiments with Pisum sativum, showed that the $^{15}N$ of nitrate, glutamate and glutamine (amide-$^{15}N$) were incorporated in the same relative manner into all amino compounds. If a GOGAT type activity were not operating, the amide group of glutamine would only donate its label to tryptophan, arginine and histidine (Miflin and Lea, 1976). Following on this lead, Lea and Miflin (1974) have demonstrated the presence of a ferredoxin-dependent GOGAT in P. sativum chloroplasts which was inactive with pyridine nucleotides but which was light-dependent. They showed that chloroplasts are capable of converting 2-oxoglutarate to glutamate in the light; also that this

reaction is not stimulated by ammonia (the substrate for GDH). A ferredoxin-dependent GOGAT has also been shown to be active in Lemna minor (Rhodes et al, 1976) in wheat (Nicklisch et al, 1976) and soybean cotyledons (Storey and Reporter, 1978). Dithionite, which presumably acts to reduce internal ferredoxin, has been shown to be an electron donor in Chorella (Lea and Miflin, 1975). The enzyme has been shown to have a Km = 2 μM for ferredoxin (Wallsgrove et al, 1977; Storey and Reporter, 1978). It appears that the GOGAT of photosynthetic plant tissue is ferredoxin-dependent while that of nonphotosynthetic tissue is NAD(P)H-dependent. This was shown by Maráchal et al (1977) in Phaseolus and Oryza where roots exhibited only NADPH-dependent GOGAT activity while the leaves showed only ferredoxin-dependent activity.

Lea and Miflin (1974) reported that the chloroplasts isolated from Pisum sativum contained GOGAT. Harel et al, (1977) found that GS and GOGAT are active in both mesophyll and bundle sheath cells in maize leaves. Rathnam and Edwards (1976) working with the $C_4$ plants Sorghum bicolor, Digitaria sanguinalis and Panicum, however, showed that 67-84% of GS and 74-100% of GOGAT activities are in the mesophyll. It has also been shown that the proplastids of tobacco cells cultured in vitro contain GS and GOGAT activities (Washitani and Sato, 1977).

The molecular weight of higher plant GOGAT is in the region of 150 000 which is much smaller than the 800 000 for that of E. coli (Miflin and Lea, 1977). Wallsgrove et al,

(1977) purified GOGAT from bean leaves and found Kms of 0,15mM for glutamine, 0,38mM for 2-oxoglutarate while other 2-oxoacids were found to be inactive. Other Km values for glutamine are: 1,43mM for pea cotyledons (Beevers and Storey, 1976), 0,6mM for pea chloroplasts (Anderson and Done, 1977), and 1,1mM for soybean cotyledons (Storey and Reporter, 1978). Other measured Km values for 2-oxoglutarate: 0,4mM (Storey and Reporter, 1978), 0,96mM (Beevers and Storey, 1976), and 0,2mM (Anderson and Done, 1977). Thus GOGAT appears to be highly specific for glutamine and 2-oxoglutarate but inactive with purified asparagine as the amide donor, (Miflin and Lea, 1975; Beevers and Storey, 1976).

## 2.4 Evidence Favouring the GS/GOGAT Pathway

The demonstration of glutamate synthase activity in higher plants (Lea and Miflin, 1974) provides an attractive system both for utilization of transported glutamine, and (together with glutamine synthetase) for the primary assimilation of ammonia (Miflin and Lea, 1976). The hypothesis which is now becoming widely accepted is commonly known as the GS/GOGAT pathway of nitrogen assimilation in plants.

However, the presence (or absence) of enzymes under assay conditions in extracts is not a sure indication of the role of the enzyme within the plant, nor can the relative flow of metabolites through alternate pathways be judged from enzyme activities (Bauer *et al*, 1977). Additional

information on the flow of nitrogen through the pools of various components of primary nitrogen assimilation has been obtained from studies using the stable isotope $^{15}N$. Other than the early studies with labelled nitrate, (Sims and Folkes, 1964; Bassham and Kirk, 1964; Kennedy, 1966a, 1966b) most of the $^{15}N$ feeding experiments indicate that glutamine is the prime recipient of newly reduced ammonia in higher plants, and not glutamate. Yemm and Willis (1956) after work on excised shoots of barley suggested that the synthesis of glutamine was an important feature of primary ammonia assimilation. In barley seedlings, after a one hour feeding with $^{15}N$-ammonium it was found that the amino and amido nitrogen of glutamine became extensively labelled (Cocking and Yemm, 1961). Also, experimenting with barley, Oji and Izawa (1971) reported that glutamine was rapidly synthesized from $^{15}N$ labelled ammonium in the roots. The $^{15}N$ abundance in glutamine was highest of all the amino acids and amides, and was considered the first assimilated form of inorganic nitrogen from ammonium-$^{15}N$ in rice seedlings (Yoneyama and Kumazawa, 1974, 1975; Arima and Kumazawa, 1975, 1977). In Helianthus leaves ammonium-$^{15}N$ was shown to be incorporated into amino compounds in the order: glutamine, glutamate, aspartate and alanine (Yoneyama and Kumazawa, 1974, 1975; Arima and Kumazawa, 1975, 1977).

Ito and Kumazawa (1976) found with feeding nitrate-$^{15}N$ to Helianthus leaves that glutamate and aspartate were higher in $^{15}N$ content, than glutamine. Since the

assimilatory pattern of nitrate was different from that of ammonium, these authors proposed that ammonium formed by the reduction of nitrate was assimilated through a pathway different from that of exogenously applied ammonium. The short feeding term data on pea leaves (Bauer et al, 1977), rice seedlings (Yoneyama and Kumazawa, 1975), and corn roots (Yoneyama et al, 1977), tend to discount this claim. Lewis (1975) and Lewis and Berry, (1975) interpreted the dominant routing of $^{15}N$ to glutamine, after several hours of feeding $^{15}NO_3^-$ to the excised shoots of Datura stramonium, to mean that glutamine is probably the initial assimilation product.

In order to investigate the route of ammonia assimilation, the responses of plants to enzyme inhibitors have been determined. Methionine sulfoximine (MSO), an analogue of the $\gamma$-glutamyl phosphate enzyme complex of GS (Tate and Meister, 1973), is a potent irreversible inhibitor of glutamine synthetase (Ronzio et al, 1969) which is without effect on glutamate dehydrogenase (Brenchley, 1973). Azaserine and 6-diazo-5-oxo-L-norvaline (DON) are anaglogues of glutamine (Lea and Norris, 1976) and inhibit all glutamine amide transer reactions, including GOGAT (Miller and Stadtman, 1972; Fowler et al, 1974; Buchanan, 1973) but do not affect GDH (Miflin and Lea, 1975). Results from experiments using $^{13}N$ and also from total pool analysis show that $N_2$ fixation is blocked by MSO at the level of $NH_4^+$, confirming the role of GS rather than GDH as the primary assimilation reaction (Stewart and Rowell, 1975; Wolk et al,

1976). The early investigations of van der Meulen and Bassham (1959) on the effect of azaserine and DON on $^{14}CO_2$ incorporation in Chlorella strongly support the involvement of GOGAT. Stewart and Rhodes (1976) using both MSO and azasarine with nitrate grown Lemna, confirm that GS is the principal enzyme for ammonia assimilation and that the synthesis of glutamate occurs via GOGAT in this plant. Both ammonia and 2-oxoglutarate increased markedly in the presence of MSO and the initial rate of ammonia accumulation is equivalent to the calculated rate of nitrate reduction. The similarity in response of relevant amino pool concentrations of Lemna (Stewart and Rhodes, 1976) and Anabaena (Stewart and Rowell, 1975) to MSO, supports the view that in nitrate grown plants, ammonia assimilation occurs via the glutamine pathway.

## 2.5 Regulation of the Major Ammonia Assimilating Enzymes

The regulation of GDH (Pateman, 1969; Joy, 1973; Shepard and Thurman, 1973) and GS (Shapiro and Stadtman, 1970; Meister, 1962; Holzer et al, 1969) has been well documented for micro-organisms. Very little interpretation and synthesis of the accumulated data on higher plants has, however, been attempted. The regulatory characteristics of bacterial GS suggest that factors other than ammonia availability may mediate the flux of ammonia through the glutamine and glutamate pathways.

ADP and 5-AMP were found to inhibit GS (Stewart and

Rhodes, 1977; Wolfolk and Stadtman, 1967) which suggests that the enzyme is subject to control by energy charge (O'Neal and Joy, 1975). 5-AMP and ADP do not show appreciable inhibition of GDH, whereas ATP has a considerable effect at concentrations as low as 0,8mM (Stewart and Rhodes, 1977). Thus, while ATP is a substrate for the GS reaction, it is an inhibitor of GDH activity.

GDH (aminating reaction) is inhibited by its end product, glutamate (Shiio and Ozaki, 1970; Stewart and Rhodes, 1977; Joy, 1973) but it is stimulated by ammonia (Sanwal and Lata, 1962; Pateman, 1969; Joy, 1969; Shepard and Thurman, 1973). In contract, GS activity is inhibited by alanine, aspartate, glycine and serine which behave as non-competitive inhibitors with respect to glutamate (Stewart and Rhodes, 1977). Growth on fairly high levels of ammonia resulted in low levels of GS activity in *E. coli* (Mecke and Holzer, 1966), *Aerobacter aerogenes* (Tempest *et al*, 1970), and *Lactobacillus arabinosus* (Ravel *et al*, 1965). This decrease in GS activity has been shown to be due to end-product inhibition (Rhodes *et al*, 1975). Rhodes and Stewart, (1977) further suggest that in *Lemna*, there is a switch from the GS/GOGAT pathway to the GDH pathway with an increased ammonia concentration. However, the magnitude of the changes are not great (approximately 50%), and the residual level of GS may be more than sufficient to cope with the observed rate of ammonia assimilation (Miflin and Lea, 1977). The

significance of this change needs further experimentation.

With a marine pseudomonad, strain $SW_2$, grown in an ammonia culture of 200 $\mu g\ cm^{-3}$, the synthesis of GS was totally repressed and the ammonia assimilation was carried out via GDH. With growth on 50 $mg\ cm^{-3}$ ammonia and all levels of nitrate used, the GS/GOGAT pathway was the major assimilatory pathway for nitrogen (Brown et al, 1972). GOGAT parallels GS in its response to increasing nitrate and ammonia concentrations in Lemna (Rhodes et al, 1976). The reduction in GS levels as a result of increasing ammonia concentrations has been shown to be an indirect response in both Lemna minor (Rhodes et al, 1975) and Candida utilis (Ferguson and Sims, 1974), the intracellular glutamine pool being the actual effector. Although the changes in levels are two to threefold, the absolute levels rarely approach zero and appreciable levels of GS, GDH and GOGAT are present under all conditions (Rhodes et al, 1976).

Any enzyme regulatory mechanism may be different between different types of higher plants, between different tissues within the same plant, or between the enzyme in the cytoplasm and in the chloroplast, (Miflin and Lea, 1977). It is difficult to extrapolate from characteristics of an enzyme determined in vitro to its behaviour in vivo. This is particularly so with multicellular organisms which exhibit a high degree of internal organisation, since such a design affords the possibility for the spatial separation of the enzymes, substrates and regulatory

metabolites. In the absence of direct measurements of effector concentrations at the site of enzyme activity, any physiological role ascribed to 'regulatory phenomena' observed *in vitro* must be somewhat speculative (Stewart and Rhodes, 1977).

## 2.6 Minor Pathways of Ammonia Assimilation

The possible routes by which ammonia could be assimilated in bacteria, include pathways involving aspartate and alanine dehydrogenases. It was suggested (Vender and Rickenberg, 1964) that ammonia assimilation, in an *E. coli* mutant lacking GDH, proceeded via aspartate ammonia lyase (aspartase). With the discovery of the GS/GOGAT pathway (Tempest *et al*, 1970), a biosynthetic role for aspartase is in doubt. Aspartase has, however, been implicated in the degradation of glutamate especially when that amino acid is used as a carbon and nitrogen source (Brown *et al*, 1974).

Alanine dehydrogenase, catalysing the reductive amination of pyruvate, has been reported to occur in bacteria (Goldman, 1959; Germano and Anderson, 1968; Johansson and Gest, 1976), fungi (Burk and Pateman, 1962), and algæ (Neilson and Doudoroff, 1973). Here too the role of alanine dehydrogenase is now assumed to be degredative, producing carbon skeltons for energy metabolism ( Brown *et al*, 1974). Neilson and Doudoroff (1973), however, have surveyed the possible enzymes of ammonia assimilation in a number of blue-green algae and concluded that alanine

dehydrogenase is assimilatory. Aspartate dehydrogenase (Santarius and Stocking, 1969) and alanine dehydrogenase (Joy, 1969) activities have been reported in plant cells, though the enzymes have yet to be purified (Bryan, 1976). Only in a few tissues is the activity of alanine and/or aspartate dehydrogenase comparable to GDH and GS, and the $^{15}N$ kinetic studies of Sims and Folkes (1964) do not support a role for them in the primary assimilation of ammonia in Candida utilis. Asparagine synthetase has been found in germinating legumes and shown to be a glutamine- and ATP-dependent enzyme, reacting with aspartate to form asparagine and glutamate (Streeter, 1977; Rognes, 1975). However, asparagine synthetase has not actually been shown to be present in nodules and the origin of the aspartate for this reaction is not known (Miflin and Lea, 1976).

Carbamyl phosphate is formed either from ammonia or glutamine and is an essential intermediate in the synthesis of arginine and pyrimidines. It seems likely that in vivo glutamine is the substrate for carbamyl phosphate synthetase in micro-organisms, due to the high Km value of this enzyme for ammonia (93mM), and it is doubtful if this enzyme contributes to ammonia assimilation (Brown et al, 1974).

## 2.7 Summary

The major pathways of newly reduced nitrogen appear to be via GS/GOGAT and GDH. The chloroplast is undoubtedly intimately involved in this process in the leaf, serving not

only as a location for these enzymes but supplying the necessary ATP and reducing power as well.

The assimilation of ammonia via GS/GOGAT provides an important alternative pathway for 2-amino production based on the results of $^{15}N$ studies, the effects of specific inhibitors on these enzymes, and their regulation and distribution. Experiments involving $^{15}N$ in conjunction with MSO, however, have yet to be performed on leaf tissue and the effect of nitrogen availability on the pathway of ammonia incorporation by higher plants requires clarification. With only a small amount of information available, the mechanism of ammonia assimilation by non-symbiotically associated roots also demands further investigation.

# CHAPTER 3

# MATERIALS AND METHODS

## 3.1 Plant Material

Helianthus annuus L. var Dwarf Sungold Sunflower seed was germinated in a Conviron Seed Germinator, Model G30, at 25°C and approximately 100% humidity on stainless steel trays containing grade number three vermiculite. When the cotyledons were about three to five centimeters high and before the first leaves had appeared, the seedlings were potted out into fifteen centimeter pots filled with number "three grade" vermiculite. Unless otherwise stated, the plants were raised on a modified Hoagland solution (Tables 1 and 2) containing 100 $\mu g\ cm^{-3}$ nitrate. Nutrient was supplied every second day. Because of difficulties encountered in trying to grow Helianthus in a greenhouse, the plants were grown entirely in a Conviron, Model E15, Growth Chamber, set at the following parameters: day temperature 21°C, night temperature 18°C, relative humidity 70%, photoperiod 14 hours, with illumination supplied by Sylvania (Canada) cool white high intensity fluorescent tubes (irradiance at the leaf surface 5,76 $mW\ cm^{-2}$), supplemented with 60W incandescent lamps (irradiance at the leaf surface 4,66 $mW\ cm^{-2}$). Helianthus was also grown hydroponically in 20$\ell$ bitumen-coated asbestos pots, with continuous aeration, and fed the nutrient solutions described in Tables 1 and 2. Because of the marked effect of temperature (Yoneyama et al,

1977) and irradiance (Tyschen, 1976) on amino acid metabolism, it was considered desirable to raise the plants and to perform the experiments under strictly controlled conditions. Four to five week old plants were used for experimentation.

## 3.2 Pretreatment - Induction Period

Whole leaves were excised under water at the first, second and third nodes above the cotyledons, and petiole-fed with either the 50 $\mu g\ cm^{-3}$ or the 300 $\mu g\ cm^{-3}$ nitrate -N feeding solution for 24 hours in the growth chamber under the conditions stated for the day regime (Section 3.1). Experimental evidence from this laboratory has shown that this period is sufficient to induce nitrate reductase to its characteristic levels for both feeding levels, without a large buildup of glutamine in the absence of such natural 'sinks' as the roots and seeds.

The inhibitor experiments were also carried out with excised leaves, which were fed at the appropriate nitrate -N level for 22 hours, followed by an additional two hours with the same nitrate -N levels but with the addition of 5mM L-methionine-DL-sulfoximine (Sigma) (Oaks, pers. comm.).

Plants to be used in root experiments were grown hydroponically in water culture solutions at the two different feeding levels to be used. For the enzyme experiments, the plants were pretreated for 2 hours with feeding solutions containing 7mM MSO prior to extraction.

TABLE 1

Macronutrients, in grams, used to make up 20ℓ of nutrient solution at three nitrate concentrations.

| Macronutrient | 50 μg N. $cm^{-3}$ | 100 μg N. $cm^{-3}$ | 300 μg N. $cm^{-3}$ |
|---|---|---|---|
| $KNO_3$ | 5,22 | 14,44 | 43,32 |
| $K_2SO_4$ | 9,40 | 3,16 | - |
| $CaCl_2.6H_2O$ | 10,00 | 10,00 | 10,00 |
| $MgSO_4$ | 4,96 | 4,96 | 4,96 |
| $NaH_2PO_4.2H_2O$ | 6,68 | 6,68 | 6,68 |

TABLE 2

Micronutrients, in milligrams, used to make up 20ℓ of nutrient solution. Micronutrients were kept at the same concentration for all nitrate feeding levels.

| Micronutrient | mg. $20ℓ^{-1}$ |
|---|---|
| $HBO_3$ | 171,6 |
| $MnSO_4.H_2O$ | 92,8 |
| $ZnSO_4.7H_2O$ | 13,2 |
| $CuSO_4.5H_2O$ | 4,8 |
| $H_2MoO_4$ | 1,2 |
| FeNaEDTA | 660,0 |

## 3.3 Methods of Isotope Feeding

The $^{15}N$-labelled potassium nitrate (99 atom percent $^{15}N$, Prochem, London U.K.) was fed in one of 3 ways:

1) through the transpiration stream (Lewis and Pate, 1973)
2) via vacuum infiltration (Ito and Kumazawa, 1976)
3) hydroponically (Arima and Kumazawa, 1977)

### 3.3.1 Transpirational Feeding of Leaves

Excised whole leaves were transferred to feeding solutions containing either 300 $\mu g$ $^{15}N$ $cm^{-3}$ or 50 $\mu g$ $^{15}N$ $cm^{-3}$ as potassium nitrate for varying lengths of time up to one hour.

This method has a serious disadvantage in that the time lag between the application of the $^{15}N$ substrate and the actual incorporation of the $^{15}N$ by the leaf limits short-term time course feeding experiments.

### 3.3.2 Vacuum Infiltration of Leaves

Vacuum infiltration of leaves of Helianthus was carried out using 400 $\mu g$ $^{15}N$ $cm^{-3}$ potassium nitrate. For the inhibitor experiments, 5mM MSO was added to this solution. Leaves were completely immersed in the infiltration solution and subjected to a vacuum of $\pm 2$ kPa for 3 min. The vacuum was released and then re-established for a further 3 min. When the vacuum was released for the second time, penetration into the intracellular spaces could be easily determined

visually. By this method, the time lag disadvantage of transpirational feeding is overcome. 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.).

The leaves were then returned to the growth chamber and transpirationally fed in the appropriate $^{15}N$-nitrate feeding solution for which they had been pretreated. These photosynthetic experiments were carried out for no longer than 60 min. It was considered that with longer times, the elevated concentration of nitrate in the intracellular tissue would lead to levels of enzyme activity uncharacteristic of the pre-treatment conditions.

### 3.3.3 Hydroponic Feeding of Roots

The roots of the intact plant were transferred, in the growth chamber, to a 300 $\mu g$ $^{15}N$ $cm^{-3}$ solution or a 50 $\mu g$ $^{15}N$ $cm^{-3}$ solution, depending on the growth solution from which they had initially been taken. On completion of the feeding period, the roots were then excised from the rest of the plant.

## 3.4 Extraction

The fresh weights of hydroponically fed roots and transpirationally fed leaves were determined immediately on harvesting. Infiltrated material was weighed prior to infiltration and the weights corrected for discarded tissue

after infiltration. Infiltrated material was killed by immersion in liquid nitrogen to maintain the strict time course nature of the experiments, and then homogenized in cold 80% ethanol (1g tissue$(50\,cm^3$ ethanol$)^{-1}$) with an Ultra Turrax homogenizer. The hydroponically-fed roots and the transpirationally-fed leaves were killed in cold 80% ethanol and homogenized. Extraction of the soluble amino compounds was carried out at $0^oC$ for 24 hours. The homogenate was then filtered through Whatman No. 1 filter paper and the ethanol extract evaporated under an airstream to a final volume of $10\,cm^3$. Chlorophyll and lipid material were removed by shaking the sample with $5\,cm^3$ petroleum ether and pouring off the petroleum ether fraction after freezing the sample.

## 3.5 Analytical Determination and Isolation of the Soluble Compounds.

Analytical determinations of soluble amino compounds were carried out using 200-500 $\mu\ell$ of extract on a Beckman Model 120C Amino Acid Analyser. Acidic and neutral amino acids were separated on a 69 cm column of Beckman PA35 spherical ion exchange resin using two lithium citrate buffers sequentially. The first buffer (0,03M Lithium; 0,05M citrate) was adjusted to pH 2,72 (Atkin and Ferdinand, 1970) and the second buffer (0,03M Lithium; 0,21M citrate) to pH 3,73 (Kedenburg, 1971). A lithium citrate buffer system was used to facilitate the separation of the amides, asparagine and glutamine. Basic amino acids were separated

on a short column of PA35 resin (12cm) with 0,35M sodium buffer at pH 5,83 (Atkin and Ferdinand, 1970; Kedenberg, 1971).

Once the amino acids were separated, they were combined with a ninhydrin solution, incubated at 100°C and optical densities recorded on a Honeywell Electronik 16 logarithmic recorder and a Beckman 125 digital integrator. The pool sizes in $\mu$ mole $cm^{-3}$ for each amino acid were calculated using the digital integrator readings corrected by specific conversion constants for each amino acid, as determined from calibration runs.

Acidic and neutral amino acids were separated and collected on a 150 cm x 1,8 cm Beckman M84 ion exchange resin column A stream divider system was used to split the eluted stream in the ratio 1 (to analytical system): 10 (to collection system). Ammonia was separated on the short column with all of the sample being collected. In both cases the eluate was collected automatically in test tubes by an LKB Ultrorac 7000 fraction collector. To determine which test tubes contained the acidic and neutral amino acids, 200 $\mu$l from each tube was spotted sequentially onto Whatman No. 41 filter paper and sprayed with ninhydrin reagent. The colour was developed after incubation in an oven at 110°C for 15 min and a visual comparison of the colour intensities obtained with the printout from the recorder to locate the amino acids. The ammonia pool was determined in a similar manner.

## 3.6 $^{15}N$ Analysis

### 3.6.1 Sample Preparation

The amino acid samples from the fraction collector were concentrated to 10cm$^3$ and were converted to ammonium sulphate by the Kjeldahl method. One mercury BDH catalyst tablet, (containing the equivalent of 0,1g mercury and 1,0g sodium sulphate) and 3cm$^3$ of nitrogen-free concentrated sulphuric acid were heated with the sample in a 50cm$^3$ micro-Kjedahl digestion flask.

Once samples were digested (after $\pm$2 h ) they were dissolved in approximately 10cm$^3$ of ammonia-free distilled water and the ammonia distilled off in a Markham micro-distillation still, after alkalisation with 15cm$^3$ of 50% sodium hydroxide. Since quantitative recovery of ammonia was not necessary, the possible loss of ammonia by binding with mercuric oxide which may be formed with the addition of the sodium hydroxide (McKenzie and Wallace, 1954) was not considered serious. The distilled ammonia was collected in 2cm$^3$ of 0,02 N hydrochloric acid and titrated against standarized 0,005 N sodium hydroxide using screened methyl red indicator. A minimum of 15 µg ammonium -N was required for the $^{15}N$ analytical system used. The optimum of 30 µg ammonium -N, however, was obtained in most cases. Samples were then acidified with 0,5cm$^3$ of 0,1N hydrochloric acid to prevent loss of ammonia, and concentrated down to a level in which 0,2 cm$^3$ contained 15-30 µg ammonia -N.

### 3.6.2 N-Discharge Tube Preparation

The method used was one described by Faust (1967) using alkaline sodium hypo-bromite solution as the oxidant. The hypobromite reacted with the ammonia under vacuum to release nitrogen gas according to the reaction:

$2NH_3 + 3\ NaOBr \longrightarrow N_2 + 3H_2O + 3NaBr$ (Kamen, 1957).

A vacuum of 0,1 Pa was achieved by a Packard mercury diffusion pump backed up by an Edwards rotary high vacuum pump. The vapour pressure of the system was reduced by the use of liquid nitrogen cold traps. The nitrogen produced by the above reaction was sealed off in a discharge tube.

### 3.6.3 $^{14}N/^{15}N$ Ratio Determination

The sealed discharge tube was placed in a Packard N-15 Statron NOI-4 atomic emission spectrophotometer (A.E.S.) for analyses. The nitrogen gas enclosed in the discharge tube was excited by a high frequency discharge which produced a red/violet colour. A blue colour indicated contamination either by water vapour or bromine. This method is based on the photoelectric recording of the intensity of the band-heads for the three molecules of nitrogen at 315,9 nm for $^{14}N^{14}N$, at 316,2 nm for $^{14}N^{15}N$, and at 316,5 nm for $^{15}N^{15}N$. A typical trace for all three molecules is shown if Fig. 1. Enrichments were calculated from the formula:

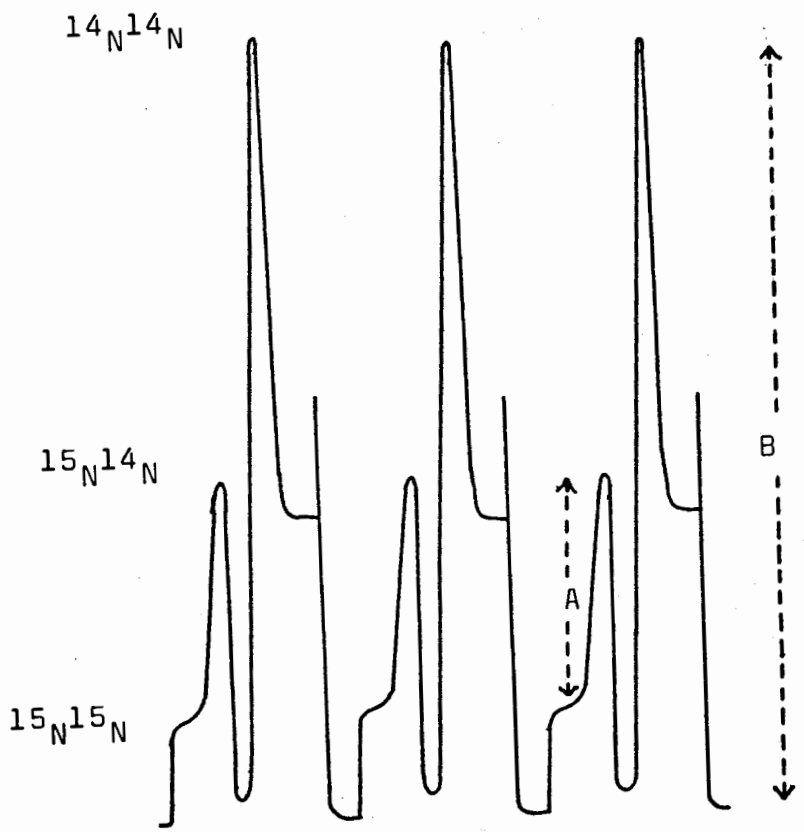


Fig. 1 : Typical traces showing good separation of the three hybrid molecules of nitrogen : $^{15}N^{15}N$, $^{15}N^{14}N$, $^{14}N^{14}N$. A and B represent the peak heights of the $^{15}N^{14}N$ and $^{14}N^{14}N$ bandheads, respectively.

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

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 were corrected using a standard calibration curve made from the set of calibration standards supplied by Packard Instruments. The percentage enrichment (A%E) in excess of the natural abundance was obtained by subtracting a natural abundance of 0,37% from the corrected percentage enrichment. Individual amino acid pool sizes expressed in $\mu$ mole $cm^{-3}$ were converted to micromoles per gram fresh weight ($\mu$ mole $gFW^{-1}$). By multiplying the pool size by the A%E value and the number of nitrogen atoms per molecule of the amino compound, the $^{15}N$ content of the particular amino pool, expressed as $\mu g$ $^{15}N$ per g fresh weight ($\mu g$ $^{15}N$ $gFW^{-1}$), could be determined. In this study, this method of expressing $^{15}N$ results was found more useful than the A%E.

### 3.7 Determination of Glutamine Amino and Amido $^{15}N$ Enrichment.

In order to determine the relative enrichments of the amino and amido nitrogen groups of glutamine, one half of the glutamine sample from the amino acid analyser was

transferred to a hydrolysis flask. Approximately 20 $cm^3$ of 6N hydrochloric acid was added to the flask and the contents bubbled with high purity nitrogen gas to remove air. The flask was then flame sealed and placed in a sand bath in an oven at 110°C for 24 h. Under these conditions the amide hydrolyses to form ammonia and glutamate.

The sample, when cooled, was distilled (Section 3.6.1) to separate the ammonia and glutamate. The glutamate portion was removed from the still and neutralized by the slow addition of concentrated hydrochloric acid. This fraction was then reduced in volume and subjected to Kjeldahl digestion and distillation. The ammonia from hydrolysis and the glutamate ammonia were analysed as amido and amino nitrogen respectively.

## 3.8 Xylem Sap Analysis

### 3.8. 1 Collection of Bleeding Sap

Plants grown in vermiculite and hydroponic tanks (Section 3.1) for 5 weeks at 50 and 300 $\mu g$ N $cm^{-3}$ (Tables 1 and 2) were excised just below the cotyledons. A collar of Tygon tubing was fitted onto the cut stem to act as a reservoir for the sap which is exuded through the action of root pressure. 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). Sap was collected in the growth cabinet over a 24 h period, under conditions of constant temperature and humidity. Samples were analysed for amino compound content (Section 3.5) and nitrate-N content (Section 3.8.2).

### 3.8.2 Determination of Nitrate-N

Samples were stored frozen prior to determinations being made. A standard nitrate solution was used to construct a calibration curve each day that determinations were done. Nitrate-N was estimated by an Orion liquid ion-exchange nitrate electrode. Samples were usually diluted with distilled water to a linear portion of the calibration curve (10-50 $\mu$g N $cm^{-3}$ nitrate).

## 3.9 Enzyme Assays for Glutamate Dehydrogenase Activity in the Leaves and Roots of Helianthus

Analysis of glutamate dehydrogenase activity was carried out on both leaves and roots of *Helianthus* grown at 100 $\mu$g N $cm^{-3}$ to determine the effect of MSO on GDH activity (Tables 1 and 2).

### 3.9.1 Extraction Procedures

#### 3.9.1.1 Leaf Tissue

The extraction buffer used was an imidazole buffer

(Rhodes et al, 1975) consisting of 0,05M imidazole-hydrochloric acid (pH 7,2), 0,5 mM ethylenediamine tetra-acetic acid and 1,0 mM dithio-threitol.

One gram of leaf material was ground with a cold mortar and pestle, 5 $cm^3$ of acid washed sand and 10 $cm^3$ of extracting buffer at 0-4 $^oC$. The ground tissue was filtered through two layers of cheese cloth and centrifuged in a Beckman J21 refrigerated centrifuge at 100 xg for 5 min at 4 $^oC$. The supernatent was transferred to another cold centrifuge tube, combined with 0,01 $cm^3$ of Triton X-50 and allowed to stand for 10 min in ice. Final centrifugation was carried out at 29 000 xg for 15 mins and the supernatant was assayed for glutamate dehydrogenase activity (Joy, 1969).

Triton X-50, a nonionic detergent of the polyethylene glycol-type, has been used extensively for the selective solubilization of biological material (Deamer and Crofts, 1967). The action of the Triton is, presumably, to remove lipids, thereby causing disintegration of membranes (Bottomley, 1970). Treatment with Triton X-50 was essential for the detection of glutamate dehydrogenase activity in the current experiments.

### 3.9.1.2 Root Tissue

The preparation of the crude extract was according to the method of Pahlich and Joy (1971) where all operations were carried out at 0-4 $^oC$. The root tissue

was washed in cold distilled water, blotted, and 2g of tissue weighed out and ground in a mortar with 5cm$^3$ of acid washed sand and 5cm$^3$ of 0,05M Tris, pH 7,5, containing 0,4M sucrose. After filtering through cheese cloth, the homogenate was centrifuged for 12 min at 1000 xg. The sediment was washed three times in grinding medium (total volume of 10 cm$^3$) and then discarded. The washings and supernatant were combined with Triton X-50 (0,1% final concentration), shaken, and allowed to stand on ice for 30 min. This solution was clarified at 25 000 xg for 10 min.

### 3.9.2 Glutamate Dehydrogenase Activity

Glutamate dehydrogenase activity was investigated in both leaf and root tissues to ascertain the effect of MSO on this enzyme. A two hour induction period with MSO was given under the conditions described for the $^{15}N$ feeding experiments (Section 3.2). The reductive amination assays of Joy (1969) for leaves and Pahlich and Joy (1971) for roots were used.

Leaf tissue assay medium contained: 1,9 cm$^3$ of buffer (see below); 0,2 cm$^3$ 1,0 M ammonium sulphate; 0,2 cm$^3$ 0,2 M 2-oxoglutarate adjusted to pH 7,5 with phosphate buffer; and 0,2 cm$^3$ of enzyme extract. The root assay mixture comprised: 2,0 cm$^3$ 0,2 M buffer (see below); 0,2 cm$^3$ 0,2 M oxoglutarate (pH 7,5); 0,2cm$^3$ 3,2 M ammonium sulphate; and 0,1 cm$^3$ of extract. Assay buffers in both

cases were: 0,2 M tris (pH 8,4) for the NADH-specific activities and 0,1 M phosphate (pH 7,5) for NADPH-specific activities. The assay mixture was then incubated at 30°C for 2 min .

The reaction was initiated by the addition of 0,5 $cm^3$ of 1,0 mM NAD(P)H and the decrease in absorbance at 340 nm was observed on a Pye Unicam SP1800 UV spectrophotometer with linear recorder. Blanks lacking 2-oxoglutarate were run for each assay.

# CHAPTER 4

# RESULTS AND DISCUSSION

## 4.1 Xylem Sap Analysis

As a preliminary study of amino compounds transported from root to leaf via the xylem, analyses of the bleeding sap from four to five week old Helianthus plants grown in vermiculite, and hydroponically, at two different nitrate concentrations, 50 and 300 $\mu g\ cm^{-3}$, were performed. The collection and analysis of bleeding sap for nitrate-N are described in Section 3.8. Analysis of free amino compounds was also performed on these samples (Section 3.5). The pool sizes for the two different growing media at both feeding levels are listed in Table 3. It was assumed that the distribution of solutes present in the bleeding sap gave a true picture of the nitrogenous compounds that normally ascend in the xylem. Although there are criticisms and disadvantages to this method of sap collection, it can yield much information as to the assimilary capacity of the roots and the distribution of nitrogen and carbon amongst the translocated solutes reaching the leaves.

Certain plants possess the ability to accumulate nutrient ions from the external medium against the concentration gradient and apparently against the electrochemical potential gradient (Bowling et al, 1966). This phenomenon is shown in Helianthus where the ratio ($NO_3^-$

xylem sap/$NO_3^-$ in the nutrient solution) is between 3,4 and 5,5 for 50 $\mu$g N $cm^{-3}$ feeding level and is between 1,9 and 2,4 for the 300 $\mu$g N $cm^{-3}$ feeding level. Probyn (1978) has shown similar ratios (between 5,8 and 6,0 for a 50 $\mu$g N $cm^{-3}$ external solution and 2,0 and 2,5 for a 200 $\mu$g N $cm^{-3}$ external solution) in Datura. Ricinus communis exhibits a ratio between 7 and 9,4 for nitrate in the xylem sap (Bowling et al, 1966).

In Helianthus, between 77% and 94% of the nitrogenous compounds being passed into the shoots from the roots, via the xylem stream, is in the form of nitrate, and is dependent on the nitrate feeding level to the roots. It is a well established fact that plants exhibit a wide spectrum of nitrate concentrations in their xylem stream, ranging from Xanthium at 98% of its sap-nitrogen as free nitrate to Lupinus with 9% of its sap as nitrogen-nitrate (Wallace and Pate, 1967). Although there is a predominance of nitrate being transported from the roots via the xylem stream to the leaves, the results in Table 3 suggest that Helianthus roots are capable of inorganic nitrogen assimilation.

The nitrate content of xylem sap has been shown to be subject to considerable changes in response to variations in the concentration of nitrate in the feeding solution (Weissman, 1964; Pate, 1967). The increase in the nitrate concentration in the feeding solution in the present experiment, showed a substantial increase in the amount of

TABLE 3 : The levels of amino compounds, nitrate and ammonia present in the xylem sap of Helianthus annuus grown in liquid culture and vermiculite at two nitrate regimes (expressed at $\mu g$ N $cm^{-3}$).

| | 50 $\mu g$ N $cm^{-3}$ Feeding Level | | 300 $\mu g$ N $cm^{-3}$ Feeding Level | |
|---|---|---|---|---|
| | Liquid culture | Vermiculite | Liquid culture | Vermiculite |
| $NO_3$ | 170,00 | 275,00 | 580,00 | 730,00 |
| $NH_4$ | 3,58 | 14,88 | 11,06 | 4,65 |
| ASP | 1,02 | 1,48 | 9,59 | 2,80 |
| THR | 0,13 | 0,21 | 0,83 | 0,32 |
| SER | 0,29 | 0,45 | 3,33 | 0,77 |
| ASG | 7,31 | 10,39 | 7,73 | 0,76 |
| GLU | 1,01 | 0,17 | 0,84 | 2,21 |
| GLN | 34,36 | 31,92 | 24,08 | 28,78 |
| GLY | 0,01 | 0,15 | 0,32 | 0,07 |
| ALA | 0,17 | 1,30 | 7,98 | 0,94 |
| VAL | 0,03 | 0,15 | T | 0,15 |
| MET | - | - | - | - |
| ILE | 0,45 | 0,63 | 0,57 | 0,25 |
| LEU | 0,03 | 0,07 | 0,25 | 0,11 |
| TYR | - | T | T | 0,01 |
| PHE | - | T | T | T |
| LYS | T | T | 1,61 | 0,53 |
| HIS | 1,47 | 0,55 | 0,62 | 3,07 |
| ARG | - | - | - | - |
| TOTAL N : | 219,86 | 337,35 | 648,81 | 775,42 |
| N as $NO_3$ : | 77% | 81.5% | 89.4% | 94% |

nitrate in the xylem stream of Helianthus (Table 3).

Amino compounds in Helianthus xylem sap with carbon/nitrogen ratios of three or less account for approximately 79-94% of the translocated amino nitrogen. These amino compounds thus carry nitrogen to the shoot with a minimum loss of carbon from the donor root, which relies heavily on the shoot for a supply of carbon skeletons for nitrogen assimilation (Dixon, 1976).

The major xylem stream amino compound at both feeding levels, and under both feeding conditions was glutamine. This is similar to the findings in many higher plants. Along with the amide glutamine, the amide asparagine and the amino acids aspartate, glutamate, alanine and histidine are the commonest N constituents of the xylem sap.

## 4.2 The Synthesis of the Major Soluble Amino Compounds

### 4.2.1 The Synthesis of Glutamine and Glutamate

#### 4.2.1.1 Nitrate-$^{15}$N Feeding Experiments

##### 4.2.1.1.1 Transpirational Stream Feeding Experiments

Leaves of Helianthus were fed through the petiole for 20, 30, and 60 min with a solution containing either 300 $\mu g\ cm^{-3}$ or 50 $\mu g\ cm^{-3}$ nitrate -$^{15}$N after a 24 hour preinduction period in 300 $\mu g\ cm^{-3}$ or 50 $\mu g\ cm^{-3}$ nitrate

-$^{14}$N respectively (see Section 3.3.1). The $^{15}$N enrichment, concentration and $^{15}$N content of six of the commonest amino compounds found in Helianthus leaves are listed in Table 4. $^{15}$N enrichment and accumulation is far greater in glutamine than in any other amino compound at all three stages of the time course, indicating the strong possibility that the GS/GOGAT pathway is operative in the leaves.

The undesirable time lag of transpirational feeding does not allow for short-term experiments. An infiltration technique was therefore used to investigate $^{15}$N incorporation at shorter time periods in subsequent experiments (see Sections 4.2.1.2 and 4.2.1.1.3).

TABLE 4 : $^{15}N$ enrichments and distribution in the commonest free amino compounds of Helianthus annuus leaves after feeding $KNO_3$ at 50 and 300 µg N $cm^{-3}$ levels for 24 hours followed by $K^{15}NO_3$ transpirational feeding for 20, 30 and 60 min.

50 µg N $cm^{-3}$ Feeding Level

| | 20 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 | 0,15 | 0,038 | 0,001 | 0,43 | 0,080 | 0,005 | 1,09 | 0,907 | 0,139 |
| ASG | 0,00 | 0,007 | - | 0,17 | 0,006 | - | 0,25 | 0,002 | - |
| SER | 0,29 | 0,076 | 0,003 | 1,80 | 0,298 | 0,075 | 1,74 | 1,478 | 0,359 |
| THR | 0,06 | 0,028 | 0,000 | 0,42 | 0,030 | 0,002 | 0,21 | 0,070 | 0,002 |
| GLN | 1,03 | 0,674 | 0,194 | 2,90 | 0,884 | 0,717 | 3,32 | 3,607 | 3,353 |
| GLU | 1,00 | 0,450 | 0,062 | 1,88 | 0,909 | 0,239 | 2,84 | 1,715 | 0,681 |

300 µg N $cm^{-3}$ Feeding Level

| | 20 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 | 0,16 | 0,071 | 0,002 | 0,43 | 0,207 | 0,012 | 0,96 | 0,241 | 0,032 |
| ASG | 0,15 | 0,016 | 0,001 | - | 0,013 | - | 0,36 | 0,020 | 0,002 |
| SER | 0,17 | 0,163 | 0,004 | 0,46 | 0,536 | 0,034 | 1,82 | 0,779 | 0,199 |
| THR | 0,15 | 0,069 | 0,001 | 0,06 | 0,052 | - | 0,42 | 0,060 | 0,004 |
| GLN | 0,42 | 0,949 | 0,112 | 1,21 | 1,783 | 0,602 | 2,81 | 3,011 | 2,368 |
| GLU | 0,27 | 0,221 | 0,008 | 0,46 | 0,639 | 0,041 | 2,03 | 1,496 | 0,426 |

4.2.1.1.2 Vacuum Infiltration Experiments

Leaves of Helianthus were infiltrated with a 400 $\mu g$ $^{15}N$ $cm^{-3}$ nitrate solution after induction for 24 hours at 2 feeding levels, (300 $\mu g$ $^{14}N$ $cm^{-3}$ and 50 $\mu g$ $^{14}N$ $cm^{-3}$ nitrate, see Section 3.3.2). Following infiltration, photosynthesis was carried out for 5, 15 and 30 min in the controlled environment chamber under the conditions described in Section 3.2. Harvesting and extraction were carried out as described in Section 3.4 and $^{15}N$ analysis of the commonest free amino compounds and ammonia was performed. The results are summarised in Table 5.

As was shown with transpirational feeding (Table 4) the routing of $^{15}N$ is predominately to glutamine at both feeding levels (Table 6). Glutamine, in spite of its large nitrogen pool, shows the highest $^{15}N$ enrichment of all the free amino compounds and also consistently shows the highest $^{15}N$ content of these compounds. The analysis of hydrolyzed glutamine from the 30 min stage of the time course at both feeding levels showed that the amido N has a far higher enrichment than the amino N (Table 5).

The ammonia pool saturates with $^{15}N$ after 5 min at an enrichment of 1,27% at the high feeding level, and after 15 min at an enrichment of 0,69% at the low feeding level, indicating the existence of more than one ammonia pool. It is probable that there is a small, actively turning over, assimilatory pool, which is regulated by the rate of

TABLE 5

$^{15}N$ enrichments of amino and amido N of glutamine of *Helianthus annuus* leaves fed at different feeding levels, ilfiltrated with $K^{15}NO_3$ (99 A%E) and allowed to photosynthesize for 30 min.

| | Feeding Level | |
|---|---|---|
| | 50 µg N $cm^{-3}$ | 300 µg N $cm^{-3}$ |
| Amido N | 6,23 A%E | 2,05 A%E |
| Amino N | 1,95 A%E | 0,45 A%E |

TABLE 6 : $^{15}N$ enrichments and distribution in the commonest free amino compounds of Helianthus annuus leaves after feeding $KNO_3$ at 50 and 300 µg N $cm^{-3}$ levels for 24 h followed by $K^{15}NO_3$ infiltration and subsequent photosynthesis for 5, 15 and 30 min periods.

50 µg N $cm^{-3}$ 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 | 0,19 | 0,390 | 0,010 | 0,51 | 0,526 | 0,038 | 2,01 | 0,595 | 0,168 |
| ASG | 0,00 | 0,046 | - | 0,14 | 0,108 | 0,003 | 0,51 | 0,071 | 0,006 |
| ALA | 0,32 | 0,737 | 0,033 | 0,54 | 0,892 | 0,067 | 1,98 | 0,734 | 0,204 |
| SER | 0,14 | 0,615 | 0,012 | 0,31 | 0,797 | 0,035 | 1,77 | 0,712 | 0,176 |
| THR | 0,05 | 0,327 | 0,003 | 0,15 | 0,539 | 0,011 | 0,20 | 0,388 | 0,011 |
| GLY | 0,21 | 0,241 | 0,007 | 0,28 | 0,234 | 0,010 | 0,51 | 0,222 | 0,016 |
| GLN | 0,69 | 1,824 | 0,352 | 1,29 | 3,829 | 1,383 | 3,28 | 2,683 | 2,464 |
| GLU | 0,66 | 2,034 | 0,188 | 0,73 | 2,413 | 0,246 | 2,81 | 1,893 | 0,745 |
| $NH_3$ | 0,50 | 1,688 | 0,118 | 0,69 | 1,718 | 0,166 | 0,63 | 1,576 | 0,139 |

300 µg N $cm^{-3}$ 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 | 0,09 | 0,758 | 0,010 | 0,15 | 0,942 | 0,020 | 0,39 | 1,079 | 0,060 |
| ASG | 0,09 | 0,757 | 0,010 | 0,28 | 0,155 | 0,006 | - | 0,156 | - |
| ALA | 0,13 | 1,433 | 0,027 | 0,26 | 1,565 | 0,057 | 0,48 | 1,760 | 0,119 |
| SER | 0,00 | 0,551 | - | 0,32 | 1,077 | 0,048 | 0,40 | 1,558 | 0,086 |
| THR | 0,00 | 0,231 | - | 0,25 | 0,303 | 0,011 | 0,21 | 0,416 | 0,013 |
| GLY | 0,10 | 0,229 | 0,003 | 0,24 | 0,306 | 0,010 | 0,35 | 0,356 | 0,017 |
| GLN | 0,27 | 8,849 | 0,669 | 0,77 | 11,081 | 2,389 | 1,03 | 10,533 | 3,038 |
| GLU | 0,10 | 3,157 | 0,044 | 0,37 | 3,523 | 0,182 | 0,59 | 3,430 | 0,283 |
| $NH_3$ | 1,27 | 1,704 | 0,303 | 0,43 | 2,213 | 0,133 | 0,21 | 1,923 | 0,057 |

nitrate reduction and the assimilation of ammonia into amino acids, and a large pool derived from amino acid metabolism in the cytosol.

Rhodes, Rendon and Stewart (1976) have shown in Lemna minor that the level of GOGAT activity is reduced with increased nitrate concentration in the feeding solution and also with increased levels of glutamine. Helianthus showed very little difference in the glutamine $^{15}N$ content between the lower and higher feeding levels, (Fig. 2), indicating its inability to respond to higher nitrate feeding levels. This was also evident in the petiole feeding experiments. The nitrophile Datura stramonium showed a 66% increase in glutamine from the lower to the higher feeding level (Probyn, 1978). In Helianthus, the decrease in glutamate incorporation of newly reduced $^{15}N$ nitrogen at the higher feeding level and the subsequent decrease in alanine, aspartate, serine and glycine enrichment (Section 4.2.2, Fig. 3 and Section 4.2.3, Fig. 4) may possibly be explained by the reduction in GOGAT activity noted above by Rhodes et al, 1976.

#### 4.2.1.1.3 Vacuum Infiltration Experiments with Methionine Sulfoximine Treated leaves

Following a 21 h enzyme induction period with nitrate feeding solutions at 2 levels (300 $\mu$g N $cm^{-3}$ and 50 $\mu$g N $cm^{-3}$ nitrate see Section 3.2), a 3 h treatment with feeding

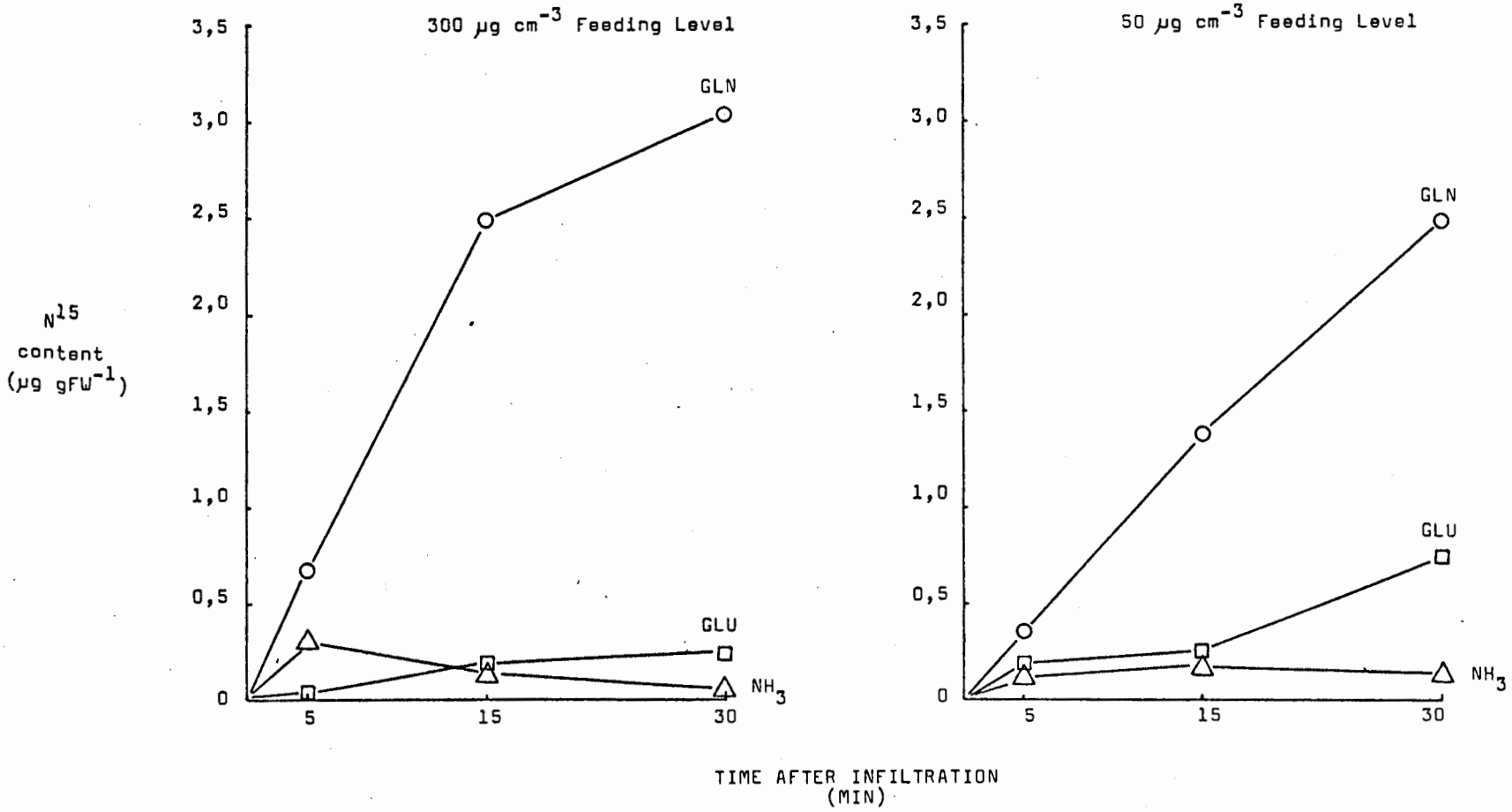


FIG. 2 : The time course of $^{15}N$ incorporation into the soluble glutamine (O——O), glutamate (□——□) and ammonia (△——△) pools of Helianthus annuus leaves. The leaves were fed via their xylem streams with either 300 µg N $cm^{-3}$ or 50 µg N $cm^{-3}$ potassium nitrate for 24 h prior to infiltration. Leaves were infiltrated with 400 µg $^{15}N$ $cm^{-3}$ nitrate and allowed to photosynthesise for 5, 15 and 30 min.

solutions containing 5mM MSO was performed prior to infiltration with 400 $\mu g$ $^{15}N$ $cm^{-3}$ containing 5mM MSO. The leaves were allowed to photosynthesize for 30 and 60 min after infiltration.

Table 7 shows the marked effect that MSO has on ammonia assimilation into the major soluble amino compounds. When compared with leaves not treated with MSO (Table 4), amino acid synthesis was shown to be virtually eliminated. The result of this supression was a very substantial increase in the size of the ammonia pool. A 24-fold and 8-fold increase in $^{15}N$ content at high and low feeding levels respectively compared with MSO untreated material, Table 4), is also apparent.

To determine whether MSO had any inhibitory effect on GDH activity, enzyme assays were run, using MSO treated and untreated leaves, as described in Section 4.3.1. These results (Table 8 ) show no difference in GDH activity between treated and untreated leaves. These findings concerning the effect of MSO on the assimilation of newly-reduced nitrogen and GDH activity suggest that nitrate-produced ammonia is virtually exclusively assimilated via the GS pathway.

Leaves treated at both feeding levels with MSO exhibit a large glutamine storage pool even though the synthesis of this amide from newly-reduced nitrogen has been completely supressed by the MSO. This probably indicates

TABLE 7 : $^{15}N$ enrichments and distribution in the commonest free amino compounds of Helianthus annuus leaves after feeding $KNO_3$ at 50 and 300 µg N $cm^{-3}$ levels for 24 h followed by $K^{15}NO_3$ and 5 mM methionine sulfoximine infiltration, and subsequent photosynthesis for 30 and 60 min periods.

50 µg N $cm^{-3}$ Feeding Level

| | 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$^{-1}$) |
| ASP | 0,04 | 0,107 | - | - | 0,087 | - |
| ASG | - | 0,041 | - | - | 0,029 | - |
| ALA | 0,09 | 0,036 | - | - | 0,018 | - |
| SER | - | 0,162 | - | - | 0,131 | - |
| THR | 0,06 | 0,301 | 0,003 | - | 0,265 | - |
| GLY | 0,05 | 0,137 | - | - | 0,136 | - |
| GLN | 0,08 | 0,262 | 0,006 | 0,03 | 0,417 | 0,004 |
| GLU | 0,02 | 0,223 | - | - | 0,296 | - |
| $NH_3$ | 1,19 | 6,683 | 1,120 | 1,85 | 10,087 | 2,618 |

300 µg N $cm^{-3}$ Feeding Level

| | 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$^{-1}$) |
| ASP | 0,01 | 0,520 | 0,001 | - | 0,273 | - |
| ASG | - | 0,153 | - | - | 0,080 | - |
| ALA | - | 0,212 | - | - | 0,182 | - |
| SER | - | 0,688 | - | - | 0,366 | - |
| THR | 0,01 | 0,343 | - | - | 0,314 | - |
| GLY | - | 0,212 | - | - | 0,175 | - |
| GLN | 0,06 | 1,765 | 0,031 | 0,02 | 1,127 | 0,009 |
| GLU | 0,02 | 1,668 | 0,005 | - | 0,911 | - |
| $NH_3$ | 1,23 | 8,038 | 1,389 | 1,52 | 10,284 | 2,184 |

the presence of an extrachloroplastic glutamine pool which is not immediately available for amino acid synthesis.

Following inhibition of the GS pathway by MSO, all of the common free amino acids exhibit considerable pool reduction with the exception of threonine. The loss from the glutamine and glutamate pools accounts for the greatest loss in terms of total nitrogen (more than 75% of the total nitrogen of the commonest free amino acids at the 30 min sample at both feeding levels). These findings suggest that glutamine and glutamate nitrogen is extensively channelled into amino acid and protein synthesis, and illuminate the important central position of GS in amino acid metabolism.

### 4.2.2 The Synthesis of Alanine and Aspartate

The time course of $^{15}N$ incorporation into aspartate and alanine is shown in Fig. 3. The complete supression of $^{15}N$ incorporation into these amino acids following MSO treatment (Table 7) precludes their participation in the direct assimilation of newly-reduced nitrogen.

It is most likely, then, that aspartate and alanine are synthesized via glutamate amino-transfer reactions. The activities of glutamate-oxaloacetate aminotransferase (GOAT) and glutamate-pyruvate aminotransferase (GPAT) have been reported from leaf chloroplasts mitochondria, peroxisomes and cytosol (Santarius and Stocking, 1969;

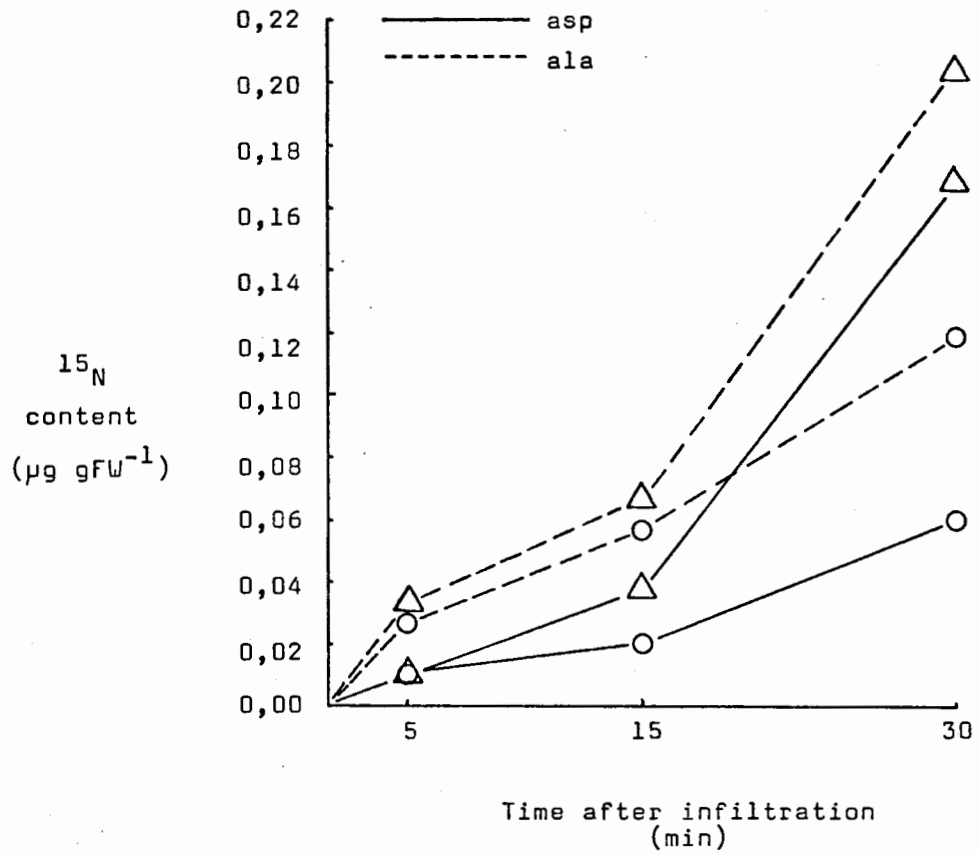


FIG. 3 : The time course of $^{15}N$ incorporation into the soluble aspartate (_____) and alanine (--------) pools of Helianthus leaves vacuum infiltrated with 400 µg $^{15}N$ $cm^{-3}$ nitrate and allowed to photosynthesise for 5, 15 and 30 min. Leaves were transpirationally fed with either 300 µg N $cm^{-3}$ (O) or 50 µg N $cm^{-3}$ (Δ) for 24 h prior to infiltration.

Huang et al, 1976). GOAT and GPAT might be extremely important in amino acid metabolism within the chloroplast in that, with the exception of leucine, 17 of the common protein amino acids can be synthesized from aspartate and alanine in secondary amino transfer reactions in the chloroplast (Kirk and Leech, 1972).

### 4.2.3 The Synthesis of Serine and Glycine

From Fig. 4 it is apparent that the accumulation of $^{15}N$ in serine is greater than that in glycine. The phosphorylated, the unphosphorylated, and the glycollate pathways of amino acid synthesis are three possible routes for serine and glycine formation (Miflin and Lea, 1977). Serine biosynthesis has been demonstrated via the phosphorylated pathway in higher plant chloroplasts (Hanford and Davies, 1958; Cheung et al, 1968). Recent tracer experiments using $^{14}C$ with maize (Chapman and Leech, 1976) and bean (Daley and Bidwell, 1977) suggest, however, that serine and phosphoserine, a proposed precursor of serine in the phosphorylated pathway, are not closely related metabolically. The photorespiratory pathways are thought to be the major routes for glycine and serine synthesis (Tolbert, 1971; Chollet and Ogren, 1975; Zelitch, 1972). Glycine, formed via the glycollate pathway in the peroxisome, can give rise to serine in the mitochondrion. Similarly, the amination of hydroxypyruvate in the

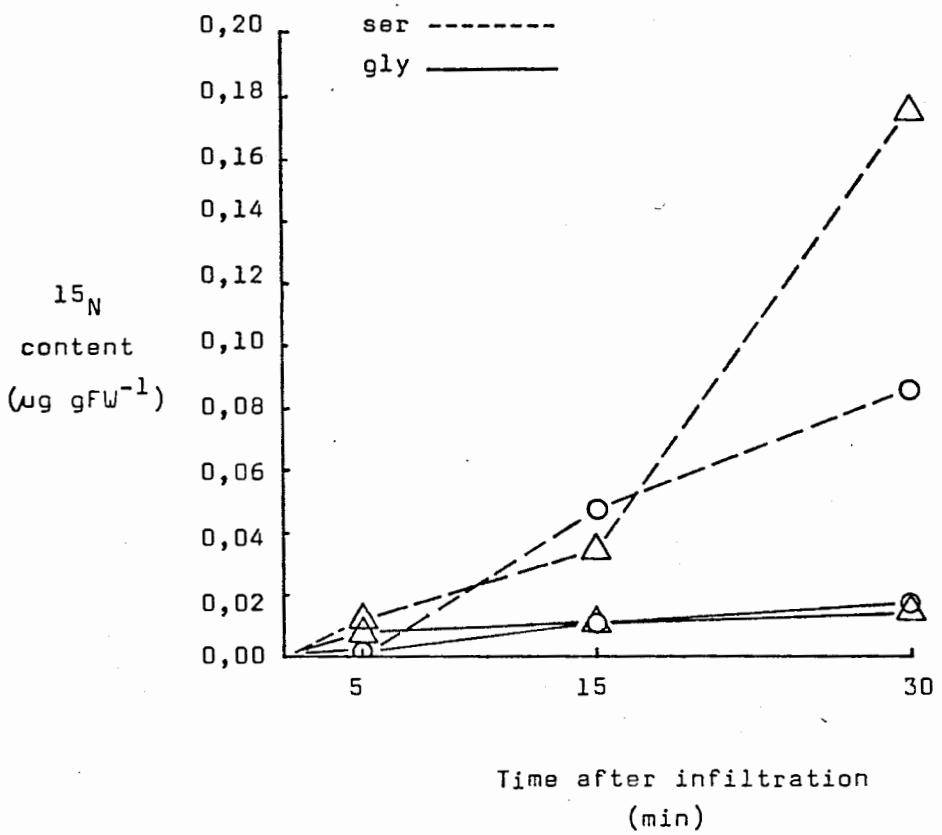


FIG. 4 : Time course of $^{15}N$ incorporation into the soluble ser (————) and gly (--------) pools of Helianthus leaves vacuum infiltrated with 400 µg $^{15}N$ $cm^{-3}$ nitrate and left to photosynthesise for 5, 15 and 30 min. Leaves were transpirationally fed either a 300 µg N $cm^{-3}$ (O) or a 50 µg N $cm^{-3}$ (△) potassium nitrate solution for 24 h prior to infiltration.

peroxisome provides an alternative method of serine and hence glycine synthesis. Thus glycine can be both a product of serine metabolism, and a precursor for serine synthesis. Since $^{15}N$ appears to accumulate at a greater rate in serine than in glycine throughout the time course, the synthesis of these amino acids probably takes place primarily via hydroxypyruvate (the unphosphorylated pathway).

### 4.2.4 The Synthesis of Asparagine

The assimilation of newly-reduced $^{15}N$ nitrogen into asparagine appears to occur only very slowly throughout the time course (Table 6). Asparagine accounts for less than 1% of the total $^{15}N$ assimilates in the commonest free amino compounds.

Originally the synthesis of asparagine was considered to occur by the direct incorporation of ammonia into aspartate at the expense of ATP (Webster and Varner, 1955; Oaks, 1967). The lack of $^{15}N$ incorporation into asparagine in the MSO experiment (Table 7) demonstrates that ammonia is not the direct substrate for asparagine synthesis in *Helianthus*.

That asparagine can be synthesized by a pathway which involves cyanide as a precursor has been demonstrated in a variety of organisms (Blumenthal-Goldschmidt *et al*, 1963;

Imada et al, 1973). Since this pathway would require a major source of cyanide, it is not considered to be a major route of asparagine synthesis. It may, however, serve as a mechanism of cyanide detoxification (Oaks and Johnson, 1972).

Asparagine synthetases capable of catalyzing the ATP-dependent amide transfer from glutamine to aspartate with the formation of asparagine and glutamate have been identified in soybean (Streeter, 1977) and lupin (Rognes, 1975). The use of aspartate labelled with $^{14}C$ (Kanamori and Matsumoto, 1974) and specific inhibitors (Lea and Fowden, 1975a) have provided evidence for the involvement of glutamine in the synthesis of asparagine, catalyzed by asparagine synthetase.

A comparison of $^{15}N$ incorporation into asparagine and aspartate (Table 6) shows a parallelism between the synthesis of these two amino compounds over the time course. From the $^{15}N$ feeding experiments and MSO treatment results it would seem that asparagine synthesis is via the asparagine synthetase pathway in Helianthus leaves.

### 4.2.5 The Synthesis of Threonine

In the present study threonine does not appear to be a primary acceptor of newly reduced nitrogen. From Table 6 it can be seen that the initial routing of $^{15}N$ into threonine is less than 1% of the total amount of $^{15}N$

incorporated into the other free amino compounds.

A comparison of the pool sizes of the major amino compounds in Tables 6 and 7 shows a reduction in all pools from the effect of MSO except in the case of threonine. The threonine pool size remains virtually unchanged. Similar results were found in Datura (Probyn, 1978). It would appear that theonine is produced outside the chloroplast via a pathway that does not involve newly reduced 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 Glutamate Dehydrogenase Enzyme Assays

Both MSO-treated and untreated leaves and roots of Helianthus were assayed for GDH activity to determine whether the GS inhibitor, MSO (Brenchley, 1973), also inhibited GDH activity. The leaves and roots were pretreated as described for the $^{15}N$ feeding experiments (Section 3.2).

In the leaves, NADPH-specific activities could not be detected with the method used (see Section 3.9). The NADH-specific activity showed little difference between the feeding levels used, as can be seen in Table 8. The MSO treated leaves showed no change in NADH-specific activity compared with untreated leaves, in accordance with other reports (Brenchley, 1973).

It can be seen from Table 8 that MSO has no effect on the NADH-dependent GDH activity of Helianthus roots.

The results of these enzyme experiments are not consistent with the involvement of GDH in the primary assimilation of newly synthesized ammonia.

TABLE 8

Effect of MSO activity on NADH-specific GDH from Helianthus annuus roots and leaves. Activities are expressed as $\mu$mole NADH oxidised $min^{-1}$ $gFW^{-1}$.

LEAVES

| | Feeding Level | |
|---|---|---|
| | 50 $\mu g\ cm^{-3}$ | 300 $\mu g\ cm^{-3}$ |
| MSO Treated | 0,037 | 0,051 |
| Untreated | 0,039 | 0,051 |

ROOTS

| | Feeding Level |
|---|---|
| | 100 $\mu g\ cm^{-3}$ |
| MSO Treated | 0,056 |
| Untreated | 0,055 |

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

There is far greater documentation of the mechanism of ammonia assimilation into leaves than its assimilation into roots. In the roots, the mitochondria (Miflin, 1970, 1974: Pahlich and Joy, 1971; Joy, 1973; Kanamori et al, 1972) are known to exhibit GDH activity. An NADH-specific GDH has been demonstrated in the roots of sunflower and soybean (Weissman, 1972a, 1972b), peas (Joy, 1973), rice (Kanamori et al, 1972), and maize (Gašparíková et al, 1976), which suggests a possible pathway for ammonia assimilation.

A GS has also been found in the roots of higher plants which has a low Km for ammonia (Kanamori and Matsumoto, 1972) and which exhibits activities some 40-fold higher than GDH (Miflin, 1974a). With the demonstration of GOGAT activity in pea roots (Fowler et al, 1974; Miflin and Lea, 1975), there is the potential for the assimilation of ammonia via the GS/GOGAT pathway in roots.

Studies involving the feeding of $^{15}$N substrates have been used to demonstrate the pathway of nitrogen assimilation in roots. Ingversen and Ivanko (1971), after feeding maize roots with $^{15}$N- nitrate for 6 hours, interpreted the routing of newly reduced nitrogen principally to glutamate to give evidence for the operation of the GDH pathway. Cocking and Yemm (1961) feeding barley roots for 1 h with $^{15}$N- ammonium found both the amido and amino

nitrogen of glutamine to have a much greater $^{15}N$ enrichment than glutamate. Detailed time course studies on rice roots with both $^{15}N$-ammonium (Yoneyama and Kumazawa, 1974; Arima and Kumazawa, 1975) and $^{15}N$-nitrate (Yoneyama and Kumazawa, 1975) showed that glutamine was initially the most heavily labelled amino compound. Corn roots showed a similar result with the amide nitrogen of glutamine being very heavily labelled with $^{15}N$ after feeding both $^{15}N$-ammonium and $^{15}N$-nitrate (Yoneyama *et al*, 1977). These results provide evidence for the operation of the GS/GOGAT pathway in roots.

One of the objects of this project was to determine which pathway of nitrogen assimilation might be in operation in *Helianthus* roots.

### 4.4.1 Hydroponic Feeding of Helianthus Roots with Nitrate-$^{15}N$

*Helianthus* plants which had been hydroponically grown in either 50 $\mu$g N $cm^{-3}$ nitrate or 300 ug N $cm^{-3}$ nitrate feeding solutions (see Tables 1 and 2, Section 3.1) were transferred to 50 $\mu$g $^{15}N$ $cm^{-3}$ nitrate and 300 ug $^{15}N$ $cm^{-3}$ nitrate feeding solutions, respectively, at the start of the experiment. A subsequent experiment with 7mM MSO included in the $^{15}N$-nitrate feeding solution was also carried out (see Section 3.3.3).

It was considered that the short term of the feeding

experiment was insufficient for the products of photosynthesis, synthesized from $^{15}N$ labelled amino compounds in the leaf, to influence the results of root $^{15}N$ metabolism. Yoneyama and Kumazawa (1974, 1975), feeding intact rice roots with $^{15}N$-ammonium for a time course of 2 h, demonstrated that up to 15 min there was virtually no $^{15}N$ enrichment in the free amino compound pools of the shoots after hydroponically feeding the roots, and very weak labelling of glutamate, aspartate, serine, glutamine and alanine after 30 min.

The incorporation of $^{15}N$ into the commonest free amino compounds of Helianthus roots fed with nitrate feeding solutions without the addition of MSO, can be seen in Table 9.

The primary recipients of the newly-reduced nitrate-$^{15}N$ were glutamine, glutamate and alanine. Threonine and glycine remained unlabelled during the time course of the $^{15}N$ experiment while aspartate, asparagine, and serine showed virtually no labelling (Table 9). Ammonia has the highest $^{15}N$ content throughout the time course of the experiment.

The pattern of glutamine as the initial acceptor of newly-reduced nitrogen shown on Table 9 is consistent with both the results of the leaf petiole feeding experiments (Table 4) and the leaf vacuum infiltration

TABLE 9 : $^{15}N$ enrichments and distribution in the commonest free amino compounds of Helianthus annuus roots after feeding $KNO_3$ at 50 and 300 µg N $cm^{-3}$ levels for 5 weeks followed by feeding $K^{15}NO_3$ at 50 and 300 µg $^{15}N$ $cm^{-3}$ levels for 15 and 30 min.

50 ug N $cm^{-3}$ Feeding Level

| | 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) |
| ASP | 0,07 | 0,229 | 0,002 | - | 0,029 | - |
| ASG | 0,01 | 0,015 | - | - | 0,040 | - |
| ALA | 0,04 | 1,657 | 0,006 | 0,33 | 0,786 | 0,036 |
| SER | - | 0,118 | - | - | 0,090 | - |
| THR | 0,02 | 0,034 | - | - | 0,032 | - |
| GLY | - | 0,015 | - | - | 0,024 | - |
| GLN | 0,34 | 0,370 | 0,036 | 0,74 | 0,481 | 0,099 |
| GLU | 0,16 | 0,618 | 0,014 | 0,47 | 0,452 | 0,029 |
| $NH_3$ | 1,47 | 0,565 | 0,116 | 2,15 | 0,735 | 0,222 |

300 ug N $cm^{-3}$ Feeding Level

| | 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) |
| ASP | - | 0,314 | - | 0,08 | 0,014 | - |
| ASG | 0,13 | 0,051 | 0,002 | 0,03 | 0,018 | - |
| ALA | - | 1,103 | - | 0,22 | 0,658 | 0,021 |
| SER | 0,08 | 0,263 | - | 0,04 | 0,125 | - |
| THR | - | 0,032 | - | 0,01 | 0,014 | - |
| GLY | - | 0,071 | - | 0,11 | 0,021 | - |
| GLN | 0,40 | 0,288 | 0,032 | 0,49 | 0,366 | 0,050 |
| GLU | 0,08 | 0,949 | 0,010 | 0,31 | 0,352 | 0,015 |
| $NH_3$ | * | 0,597 | * | 2,59 | 1,754 | 0,637 |

* Sample lost

experiments (Table 6) indicating that the GS/GOGAT pathway is operative in both leaf and root. The low $^{15}N$ labelling of glutamate does not lend support to the concept that a GDH pathway is active in the roots of Helianthus.

Helianthus roots appear to have a much slower rate of $^{15}N$ incorporation than do the leaves. The lack of $^{15}N$ labelling of alanine, serine and glycine throughout the time course is in contrast to their high labelling in the leaf, where their production is obviously of photosynthetic origin. As in Helianthus leaves, aspartate, asparagine and threonine do not appear to be important early recipients of the initial metabolism of newly-reduced nitrogen.

### 4.4.2 Hydroponic Feeding with Nitrate-$^{15}N$ Solution containing Methionine Sulfoximine

Helianthus roots were fed at the two feeding levels with nitrate-$^{15}N$ solutions containing 7mM MSO (Section 3.3.3) to determine the dependence of ammonia assimilation on GS and the possible involvement of GDH in this process. From Table 10 it can be seen that the assimilation of newly-reduced $^{15}N$ into amino compounds was virtually completely supressed, while accumulation in ammonia increased. The slight enrichment into some amino compounds seen in Table 10 may be due to GS/GOGAT activity prior to

TABLE 10 :- $^{15}N$ enrichments and distribution in the commonest free amino compounds of Helianthus annuus roots after growth in liquid culture at two nitrate regimes, feeding $K^{15}NO_3$ and 7 mM methionine sulfoximine at 50 and 300 µg N $cm^{-3}$ and subsequent photosynthesis of the intact plants for 15 and 30 min periods.

50 µg N $cm^{-3}$ Feeding Level

| | 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) |
| ASP | - | 0,113 | - | - | 0,077 | - |
| ASG | 0,01 | 0,054 | - | - | 0,046 | - |
| ALA | 0,01 | 0,535 | - | - | 0,721 | - |
| SER | - | 0,208 | - | - | 0,195 | - |
| THR | 0,01 | 0,071 | - | - | 0,038 | - |
| GLY | - | 0,096 | - | - | 0,046 | - |
| GLN | - | 0,033 | - | - | 0,005 | - |
| GLU | - | 0,738 | - | - | 0,685 | - |
| $NH_3$ | 1,85 | 2,095 | 0,539 | 2,87 | 1,415 | 0,569 |

300 µg N $cm^{-3}$ Feeding Level

| | 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) |
| ASP | 0,02 | 0,182 | - | 0,03 | 0,050 | - |
| ASG | - | 0,041 | - | 0,05 | 0,046 | - |
| ALA | - | 0,267 | - | - | 0,402 | - |
| SER | - | 0,171 | - | - | 0,114 | - |
| THR | - | 0,063 | - | 0,01 | 0,039 | - |
| GLY | 0,02 | 0,061 | - | - | 0,054 | - |
| GLN | 0,19 | 0,006 | - | - | 0,007 | - |
| GLU | 0,01 | 0,675 | - | - | 0,394 | - |
| $NH_3$ | 2,75 | 1,581 | 0,608 | 2,48 | 1,658 | 0,575 |

the inhibitor MSO reaching the active site for GS. This is similar to the effect reported for the leaves (Table 7). A comparison of Tables 9 and 10 shows that the glutamine pool in MSO treated roots was reduced to less than 2% of the pool size in the untreated roots in the 50 $\mu$g N $cm^{-3}$ and 300 $\mu$g N $cm^{-3}$ samples at 30 min. Similar results were found in rice roots after MSO treatment (Arima and Kumazawa, 1977). This demonstrates that, unlike the case for the leaves (Section 4.2.1.1.3), there appears to be no large storage pool of glutamine in the roots.

The complets supression of $^{15}N$ incorporation into glutamate does not support an active role for GDH in the primary assimilation of newly-reduced ammonia. GDH activity was assayed in the roots following pretreatment described in Section 3.2. As with the leaves, only a NADH-dependent GDH activity was found and it remained unaffected by MSO treatment at both feeding levels (Table 8). These results demonstrate the probable exclusive operation of the glutamine synthetase/glutamate synthase pathway and preclude any role for glutamate dehydrogenase in the primary assimilation of newly-reduced $^{15}N$ in Helianthus roots.

# CHAPTER 5

# CONCLUSIONS

In Helianthus leaves, the pathway for 2-amino production from newly-reduced nitrogen is apparently via the GS/GOGAT pathway. Even under conditions of elevated ammonia concentrations, NADH-specific GDH does not appear to contribute significantly to the assimilation of ammonia produced from newly-reduced nitrate.

It appears that in Helianthus leaves, the first recipient of newly-reduced nitrogen is glutamine, which donates its nitrogen to glutamate via the GOGAT pathway. Alanine, aspartate, serine and glycine, which are important in the early metabolism of newly-reduced nitrogen, are most likely synthesized in amino transfer reactions and not from direct amination reactions.

The higher feeding concentration appears to have an inhibitory effect on the GS/GOGAT pathway in Helianthus. At the higher feeding level, glutamine $^{15}N$ enrichment is only slightly higher than at the lower feeding level, and $^{15}N$ accumulation in glutamate and amino compounds resulting from glutamate amino transferase activity, is decreased.

The root of Helianthus, is capable of nitrogen assimilation into soluble amino compounds. This assimilation appears to proceed via the GS/GOGAT pathway,

as is the case for the leaves. As in leaves, the activity of NADH-specific GDH is unaffected by ammonia concentrations or the presence of MSO.

The Helianthus root appears to have a much slower rate of incorporation of newly-reduced nitrogen into the soluble amino compounds than do the leaves. Apart from glutamine and glutamate, the other most significant amino compound in the early metabolism of newly-reduced nitrogen is alanine.

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