A ^{15}N STUDY OF THE EFFECTS OF NITRATE, AMMONIUM AND NITRATE PLUS AMMONIUM NUTRITION ON NITROGEN ASSIMILATION IN *HORDEUM VULGARE* L.

by

Susan G. Chadwick

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ABBREVIATIONS AND SYMBOLS

A%E	atom per cent excess
ATP	adenosine triphosphate
GDH	glutamate dehydrogenase
GOGAT	glutamate synthase
GS	glutamine synthetase
MSO	methionine sulfoximine
NADH	nicotinamide adenine dinucleotide (reduced)
NiR	nitrite reductase
NR	nitrate reductase

ABSTRACT

A review of the recent literature concerning the assimilation and utilisation of nitrate and ammonium nitrogen within plants has been presented.

Barley plants (Hordeum vulgare L.cv. Clipper) were grown hydroponically under controlled environmental conditions. The aerated nutrient solutions contained 2mM inorganic ^{14}N supplied as either nitrate alone, ammonium alone, a 1:1 nitrate plus ammonium mixture or a 3:1 nitrate plus ammonium mixture. After 20 days of growth ^{15}N nutrient solutions were substituted. The plant material was harvested four and eight hours after the commencement of the ^{15}N feeding experiment and prepared for analysis. Xylem sap was also collected for a period of one hour beginning half an hour before each harvest and continuing for half an hour after harvesting. Separate batches of plants were used for harvesting and sap collection.

In nitrate-fed plants the shoot was shown to be the main organ of nitrate assimilation. Xylem sap analysis indicated that 66% of the ^{15}N supply to the shoot was in the form of nitrate and the majority of the absorbed and assimilated ^{15}N was located in this region. In ammonium-fed plants, however, ^{15}N-ammonium accumulated in the root with only a very small amount detectable in the xylem sap. Some 93% of the ^{15}N exported from root to shoot in the xylem stream was in the form of organic nitrogen (mainly glutamine). This indicated that the root was the major organ of ammonium assimilation and that the shoot was the main destination of root assimilated nitrogen.

The different rôles of the root and shoot in response to nitrate and

ammonium nutrition were reflected in plants from the mixed nitrogen feeding regimes. At the 8h harvest there were higher concentrations of ^{15}N-nitrate in the shoot than in the root, whereas ^{15}N-ammonium occurred at higher levels in the root. In plants from the 1:1 mixed feed a suppression of nitrate ion absorption by ammonium was indicated.

In terms of the rate of ^{15}N incorporation into organic nitrogen the 1:1 mixed feed plants showed the highest figures (24.8 $\mu g\ h^{-1}$ per plant). The ammonium-fed plants, with a rate of 22.4 $\mu g\ h^{-1}$ per plant were followed closely by the 3:1 mixed feed plants (21.9 $\mu g\ h^{-1}$ per plant). The nitrate-fed plants had the lowest rate (5.6 $\mu g\ h^{-1}$ per plant) and in this investigation this is ascribed mainly to a decreased uptake of the nitrate ion in comparison to the ammonium ion. Plants from both the 1:1 and 3:1 mixed feed were larger than those from the other feeding regimes and were very robust. The nitrate-fed plants were small with very erect, fibrous stems in contrast to the ammonium plants which had soft stems with a tendency to lodge.

Enzyme assays were also carried out to determine whether the enzyme glutamate dehydrogenase (GDH; EC 1.4.1.3) played a significant rôle in ammonium assimilation in the roots of barley plants.

The incorporation of ^{15}N-ammonium into amino compounds in barley roots was investigated in two ways:

i. When both the glutamine synthetase (GS; EC 6.3.1.2) / glutamate synthase (GOGAT) pathway and the GDH pathway were potentially operative.

ii. When only the GDH pathway was capable of operating after the GS-GOGAT pathway was inactivated using the GS inhibitor methionine sulfoximine (MSO).

Root enzyme assays demonstrated that GS is completely inhibited by

MSO treatment. The activity of GDH, however, is not affected but free amino compounds remain unlabelled and ^{15}N-ammonium accumulates in the root when MSO is fed to barley via the ^{15}N-ammonium feeding solution. After feeding ^{15}N-glutamine in the presence of MSO the free amino compounds of the root became labelled. This indicated that MSO did not interfere substantially with nitrogen assimilation after the formation of glutamine.

These results show that in barley roots ammonium is assimilated entirely via the GS-GOGAT pathway and that GDH plays a negligible rôle in this process.

CHAPTER 1

INTRODUCTION

With the present world population standing at an estimated 4,800 million the need to develop ways of increasing the nutritional quality and quantity of crop plants is at a premium, and since protein is the major nutritional limitation in many human diets the ability to manipulate the protein status of crop plants would be very advantageous. Research into the nitrogen nutrition of plants has shown that their protein and mineral composition can be modified by controlling the form of nitrogen supplied (De Kock, 1970; Cox and Reisenauer, 1973; Ikeda, Yamada and Harada, 1974).

It is accepted that the element nitrogen plays a crucial rôle in plant metabolism and that its absence as a soil nutrient limits growth. Under natural conditions the main sources of nitrogen utilised by the plant are the ions ammonium and nitrate but the extent to which these ions are absorbed, and the manner in which they are assimilated, varies considerably with the plant species.

Nitrate, which forms the most readily available source of nitrogen to the plant in the field, has to be reduced to ammonium within the plant before it can be incorporated into organic compounds. The enzymes catalysing nitrate reduction are nitrate reductase (reduced nicotinamide adenine dinucleotide nitrate oxidoreductase, E.C.1.6.6.1.) and nitrite reductase (reduced benzyl-viologen nitrite oxidoreductase, E.C. 1.7.7.1.).

Once ammonium is absorbed, however, it can be directly incorporated into an organic form via the combined action of the enzymes glutamine synthetase (E.C. 6.3.1.2.) and glutamate synthase (E.C. 1.4.1.13.).

By using ^{15}N feeding techniques, xylem sap analysis and enzyme assays it has been shown that the roots of barley plants predominate as sites of ammonium assimilation to glutamate, whereas nitrate reduction takes place mainly in the shoot (Muhammad and Kumazawa, 1974; Lewis, Watson and Hewitt, 1982). This division of assimilatory activity between roots and shoots may be partly responsible for the increased growth and yield observed in plants receiving a mixture of nitrate and ammonium as their nitrogen source. In addition, the reduced energy requirement achieved when ammonium is used instead of nitrate for protein synthesis, may also be an important factor.

The purpose of this project was to investigate the influence of nitrate and ammonium, supplied individually as sole nitrogen sources or in 1:1 or 3:1 mixtures, on the assimilation and utilisation of nitrogen in young barley plants (Hordeum vulgare L. cv. Clipper).

Using ^{15}N feeding techniques and enzyme assays, the relative absorption rates of the two ions and their subsequent transportation, compartmentalisation and incorporation into organic compounds within the plant were studied.

CHAPTER 2

LITERATURE REVIEW

2.1 Factors Affecting Ammonium and Nitrate Absorption

The absorption of the ions ammonium and nitrate have been shown to be influenced by pH, temperature, carbohydrate supply, nitrogen concentration and plant species.

2.1.1 pH

It has been observed that ammonium absorption is enhanced with increasing pH (Cox and Reisenauer, 1973), whereas nitrate uptake tends to decrease with increasing pH (Warncke and Barber, 1973). As plants take up ammonium or nitrate, however, they change the pH of their growth medium; a nutrient solution containing anionic nitrate becomes more alkaline, whereas a nutrient solution containing cationic ammonium becomes more acidic. These results have been shown for a variety of plant species (Chen, 1960; Karim and Vlamis, 1962; Kirkby and Hughes, 1970). As an illustration of these effects Chen (1960) reported that rice plants absorbed the maximum amount of ammonium from a medium buffered at pH 8, while the maximum absorption of nitrate occurred at pH 4. He also concluded that when no pH correction was applied the medium around young rice plants tended towards a pH of 7.2 during nitrate nutrition, whereas a pH of 4.3 was produced during ammonium nutrition.

Changes in external pH are due to the excretion of hydroxyl or bicarbonate ions during nitrate uptake (Wallace and Mueller,1963) and of hydrogen ions during ammonium uptake. In an investigation into the relationships between nitrate reduction, protein synthesis and malate accumulation in corn (*Zea mays* L.), tobacco (*Nicotiana rustica* L.) and barley (*Hordeum vulgare* L. cv. Delta), a model was proposed by Ben Zioni *et al*., (1970 & 1971) relating nitrate uptake by roots and the efflux of bicarbonate ions from the roots into the external medium. This model was founded on the basic premise that the reduction of nitrate in leaves establishes an 'excess' of cations in the tissue and that this is neutralised by the synthesis of malate. The reduction of nitrate was shown to be stoichiometric with the synthesis of malate. The bicarbonate in the root is suggested to be mainly derived from the oxidation of this malate after it has been transported from the shoot to the root in the form of potassium malate. Potassium bicarbonate is then exchanged for potassium nitrate taken up from the external nutrient solution. In this hypothesis, potassium ions are an important component in this shuttle system between root and shoot, accompanying nitrate up to the shoot for reduction and malate down to the root for oxidation.

Hydroxyl ion excretion has also been observed to occur upon nitrate uptake (Eisele and Ullrich, 1975), and a variation in the theoretical model of Ben Zioni *et al*., was put foreward by Kirkby in 1974 accounting for this. It was suggested that after the oxidation of malate, carbon would be retained and hydroxyl ions excreted instead of bicarbonate in exchange for nitrate ions taken up from the external medium.

2.1.2 Temperature

In nutrient solution nitrate uptake is affected more by lower temperatures than is the uptake of ammonium (Zsoldos, 1971; Frota and Tucker, 1972). Ammonium absorption is greater than nitrate at 8°C and reaches a maximum at a media temperature of 25°C; nitrate absorption however, becomes greater than ammonium at 23°C and increases up to 35° C (Lycklama, 1963; Frota and Tucker, 1972). It was noted, by Williams and Vlamis (1962), that almost no nitrate absorption occurred at 13°C but absorption of phosphate, sulphate, potassium, calcium, magnesium and ammonium continued. This reduced nitrate uptake at low temperatures may be linked to the reduced activity of an enzyme-controlled uptake system. The initial low rate of nitrate uptake observed when nitrogen deficient seedlings are transferred to a nitrate-containing medium (Jackson _et al._, 1973; Blevins, Hiatt and Lowe, 1974; Rao & Rains, 1976a,b), and the following increase in the rate of nitrate uptake to a maximum value (Ashley _et al._, 1975) indicate that such a system may be operating. In addition the inhibition of the subsequent accelerated rate of uptake by inhibitors of protein synthesis, such as cycloheximide, has been interpreted as evidence for the involvement of a specific nitrate transport system, or permease, in nitrate absorption (Heimer & Filner, 1970; Jackson _et al._, 1973).

2.1.3 Carbohydrate Supply

The assimilation of ammonium into organic molecules (and also nitrate since this ion is reduced to ammonium after its absorption) depends on an adequate supply of carbon skeletons. This incorporation of ammonium into amino acids and amides can be seen as a detoxification process, as high levels of ammonium in plants become toxic. That a relationship exists between carbohydrate supply and ammonium and nitrate nutrition has been demonstrated (Carrodus, 1966; Michael, Martin and Owassia, 1970; Ojima and Ohira, 1976). The high ammonium uptake by young potato plants grown in a nutrient solution has been shown to be a result of the carbohydrate supply from the parent tuber (Michael *et al*, 1970). When the parent tuber is removed, the young plants absorb relatively more nitrate than ammonium. These workers also showed that by ringing bean plants (*Phaseolus vulgaris*), to remove the phloem and so reduce the carbohydrate supply to the root, the uptake of nitrogen was reduced when compared to intact plants. The uptake of ammonium was affected to a greater extent than that of nitrate.

A phenomenon that must be mentioned here is the apparent stimulation of photosynthesis in intact chloroplasts by ammonium, when supplied at concentrations well below those required for uncoupling of photophosphorylation (De Benedetti, Forti, Garlaschi and Rosa, 1976). This may be related to the need for carbon skeletons under ammonium nutrition. These workers concluded that the mechanism of stimulation of photosynthesis by ammonium, was through the activation of the ribulose bisphosphate (RUBP) carboxylase reaction.

2.1.4 Nitrogen Concentration

In an investigation into nitrogen uptake in corn (*Zea mays* L.), Warncke and Barber (1973) calculated root uptake coefficients. This allowed the uptake of total nitrogen, nitrate and ammonium per cm^2

of root surface to be related to their concentration in the nutrient solution. They reported that as nitrogen concentration increased the nitrogen uptake coefficient decreased, and that this was linked to the observation that the efficiency of nitrogen uptake decreases with increasing nitrogen concentration. Similarly, the nitrate and ammonium uptake coefficients decreased as their respective concentration increased. These values agreed with those for corn seedlings grown in nutrient solutions containing only nitrate-nitrogen (Warncke and Barber, 1974). At nitrate concentrations of 100, 1000 and 5000μM, the coefficients were 10.82, 1.56 and 0.39 x 10^{-5} cm s^{-1} respectively.

Cox and Reisenauer (1973) reported that in wheat, the intake rates of nitrate and ammonium, at low nitrogen concentrations, were related to the concentration of the ion in the root medium. The rate of nitrate intake increased until the nitrate concentration in the root medium approached 90uM. The rate of intake of ammonium, however, decreased as ammonium toxicity reduced growth. Rates of ammonium intake in excess of 98μM g^{-1} h^{-1} apparently produced symptoms of ammonium toxicity.

2.1.5 Plant Species

The availability of nitrate and ammonium ions can vary considerably from one plant habitat to another. This makes it necessary for species from different habitats to exhibit different nitrogen uptake and assimilation strategies.

Ingestad (1976) made a study of the nutrient requirements of three ecologically different plants under nitrate, ammonium or mixed nitrate and ammonium nutrition at pH 4.0-5.2. In general the plants, Betula verrucosa

Ehrh., (ecologically widespread), Cucumis sativus L. (a calcicole), and Vaccinium vitis idaea L. (a calcifuge), responded equally to the three nitrogen sources except that growth was reduced in C. sativus at high ammonium levels, and in V. vitis idaea under high levels of nitrate.

The regulation of ammonium and nitrate uptake by the plants was found to be related to their soil environment adaptations. When high ionic concentrations of ammonium and nitrate were supplied, the uptake of ammonium by C. sativus increased with increasing concentration, while nitrate did not. The reverse was shown by B. verrucosa. In the calcifuge, V. vitis idaea however, both ammonium and nitrate increased with increasing ionic concentration. These results were in agreement with the fact that V. vitis idaea and B. verrucosa can grow in areas of low nutrient availability (although B. verrucosa also grows in rich soils) using ammonium as the main nitrogen source. These results emphasise the difference in response of plants with different ecological distributions with regard to their uptake of nitrogen.

Bogner (1968) carried out a more extensive survey to determine the response of 84 woodland species to nitrogen supplied as nitrate and ammonium alone or in some combination. As in the case of many crop plants (Hewitt, 1966) Bogner observed better growth of many of the species on a mixture of nitrate and ammonium. Although the majority of plants have the ability to absorb and assimilate both nitrate and ammonium it is common for a preference to be shown for one or other of the ions.

2.2 Enzymes of Nitrate Reduction

Assimilatory nitrate reduction involves the reduction of nitrate, via nitrite, to ammonium and its subsequent incorporation into organic nitrogenous compounds. That nitrate incorporation into amides and amino acids takes place after the reduction of nitrate to ammonium, has been shown by Yoneyama and Kumazawa (1974, 1975). They demonstrated that the pattern of nitrate assimilation, to the level of proteins, was very similar to that of ammonium assimilation.

Nitrate reduction is catalysed by the action of two enzymes. Nitrate reductase (NR) reduces nitrate to nitrite, and nitrite reductase (NiR) reduces nitrite to ammonium; the overall reaction requiring 8 electrons. The electron donor for NR in higher plants, in the majority of cases, is NADH (Beevers and Hageman, 1969), although NADPH and reduced flavins may also act as electron donors (Oji and Izawa, 1969). Molybdenum appears to be an important component of the enzyme (Notton and Hewitt, 1971) but there is also evidence that it may also contain a heme-iron (Amy and Garret, 1974).

Nitrite reductase has been located in both chloroplasts (Beevers and Hageman, 1969; Magalhaes, Neyra and Hageman, 1974), and root plastids (Dalling, Tolbert and Hageman, 1972). Ferredoxin is the electron donor for nitrite reductase (Hageman, Cresswell & Hewitt, 1962). The reducing power necessary for the reduction of ferredoxin may be either supplied photochemically during non-cyclic photophosphorylation, or by NADPH in the presence of ferredoxin NADP reductase. Highly purified preparations of nitrite reductase can catalyse the stoichiometric reduction of nitrite to ammonia. In addition it can also reduce hydroxylamine (Hewitt, 1975). Although this is a highly toxic compound, it may be a very short-lived intermediate in the nitrite reduction reaction.

2.2.1 Control of Nitrate-Nitrogen Assimilation

Although the presence of substrate-inducible enzymes is a widespread phenomenon in bacteria (Mandelstam, 1971) there are relatively few examples in higher plants (Stewart and Rhodes, 1977a). It is now well known that nitrate reductase is an enzyme which is inducible by nitrate nitrogen, although it has also been suggested that in bean cotyledons (*P. vulgaris* L.) nitrite, and not nitrate, is the inducer of nitrate and nitrite reductase enzymes (Lips, Kaplan and Ruth-Bejerano, 1973).

Many workers have implicated light in the reduction of nitrate nitrogen (Travis *et al*, 1970; Berner, 1971a,b; Sawhney and Nair, 1972; Sluiters-scholten, 1973). Other studies however, have shown that roots (Sarkissian and Fowler, 1974) and other non-green tissues (Wray and Filner, 1970) are able to reduce nitrate. A scheme put forward by Klepper, Flesher and Hageman (1971) suggests that photosynthetically produced sugars are transported from the chloroplast to the cytoplasm and metabolised by glycolysis. The NADH produced from the oxidation of glyceraldehyde-3-phosphate during this process provides a source of electrons for nitrate reduction. It was also reported by Ben Zioni *et al* (1970) that the reduction of nitrate was correlated to the synthesis of malate. It is therefore possible that the NADH produced as a result of the oxidation of malate to oxaloacetate is another source of the electron donor which is required for nitrate reduction.

The inhibition or repression of nitrate reductase formation can be brought about by products of nitrogen assimilation, such as ammonium, amides and amino acids. This type of control exerted by ammonium has been demonstrated in excised barley and maize roots (Smith and Thompson, 1971; Oaks, Aslam and Boesel, 1977). In leaves however, it is reported that ammonium seldom produces such an effect (Afridi and Hewitt, 1964; Beevers _et al_, 1965; Stewart _et al_, 1974). The effects of ammonium on nitrate distribution and flux, and on nitrate reductase activity has been investigated in barley, wheat and in maize by Minotti, Craig and Jackson (1969), Schrader _et al_.,(1972), Rao and Rains (1978) and Lewis _et al_.,(1982). The results indicated that nitrate uptake was inhibited by ammonium. This led Lewis _et al_. (1982) to conclude that the observed decrease in nitrate reductase activity in roots and leaves of barley was due to antagonism of nitrate uptake rather than repression by ammonium.

The differential sensitivity of root and shoot nitrate reductases to repression by amino compounds may be related to the contribution of root and shoot systems in plant nitrate assimilation. This sensitivity of the root enzyme to repression could be important to plants in which the shoot system is the principle site of nitrate assimilation.

2.3 Enzymes of Ammonia Assimilation

2.3.1 Glutamate Dehydrogenase

This enzyme is widely distributed in higher plants. In general glutamate dehydrogenase (GDH) exhibits activity with both NADH and NADPH as electron donors. Two forms appear to be present in some plants; one which shows greater activity with NADH than with NADPH, and one which is apparently equally active with both (Lea and Thurman, 1972). Lee and Stewart (1978) examined a range of plant species for ammonium assimilating enzymes. In shoot tissue of all the species it was possible to detect NADH-dependent GDH. When NADPH replaced NADH as the electron donor, GDH remained active in most of the species analysed, but in general, the activity with NADPH was less than 25% of that with NADH. These workers also observed that in 70% of the shoot tissues studied, the specific activity of GDH was less than half that of glutamine synthetase. In contrast, over 60% of root tissue examined had GDH levels at least twice those of glutamine synthetase.

Despite the high GDH activity noted in either, or both, the root and shoot systems of some species, the kinetic characteristics of GDH suggest that a major role for this enzyme in ammonium assimilation is unlikely. Plant glutamate dehydrogenases generally have a low affinity for ammonium ions with K_m values in excess of 1×10^{-2}M being reported (Pahlich and Joy, 1971; Teixiera and Davies, 1974).

2.3.2 Glutamine Synthetase

This enzyme has a high affinity for ammonium ions, the K_m values generally being in the range 1-2 x 10^{-5} (O'Neal and Joy, 1974; Stewart and Rhodes, 1977). The combined action of glutamine synthetase and glutamate synthase constitutes the main route by which ammonium is assimilated in higher plants. The net result, one glutamate molecule, is the same as that produced by glutamate dehydrogenase but the route involving glutamine synthetase involves the expenditure of energy in the form of ATP. The enzyme has a requirement for a divalent metal cation and while Mn^{2+}, Co^{2+} and Ni^{2+} can satisfy this, Mg^{2+} is regarded as being the most important physiological co-factor.

In addition to catalysing the synthesis of glutamine the enzyme will also catalyse the so-called transferase reaction:

$$\text{L-Glutamine} + NH_2OH \xrightarrow[\text{ADP; } AsO_4]{Me^{2+}} 2\ \text{L-glutamyl hydroxamate} + NH_3$$

This reaction can be up to 20 times more active than the synthetase assay and consequently is used to determine the glutamine synthetase activity. The transferase reaction has no known physiological function.

2.3.3 Glutamate Synthase

Two forms of the enzyme have been reported in higher plants. One of these utilises reduced ferredoxin as the physiological electron donor and has been found in both chlorophyllous and non-green tissue (Lea and Miflin, 1974; Miflin and Lea, 1975). The other form of the enzyme is pyridine-nucleotide-linked, exhibiting activity with both NADH and NADPH (Dougall, 1974; Fowler *et al.*, 1974). Following the discovery of this enzyme there was some doubt as to the substrate specificity of NAD(P)H-linked glutamate synthase. Crude cell preparations apparently exhibited activity with asparagine (Dougall, 1974; Fowler *et al.*, 1974). Miflin and Lea (1976), however, demonstrated that this lack of substrate specificity resulted in part from the use of impure asparagine.

2.4 Pathway of Ammonia Assimilation

The assimilation of ammonia in higher plants is now known to take place via the combined action of the enzymes glutamine synthetase (GS) and glutamate synthase (GOGAT). The reactions catalysed by these two enzymes are:

$$\text{L-glutamate} + NH_3 + \text{ATP} \xrightarrow{Me^{2+}} \text{L-glutamine} + \text{ADP} + P_i$$

and

$$\text{L-glutamine} + \text{2-oxoglutarate} + \text{NAD(P)H} + H^+ \longrightarrow \text{2 L-glutamate} + \text{NAD(P)} + H_2O$$

or

$$\text{L-glutamine} + \text{2-oxoglutarate} + \text{FdH} \longrightarrow \text{2 L-glutamate} + \text{Fd}$$

Prior to the discovery of GOGAT activity in higher plants (Dougall, 1974; Fowler et al., 1974; Lea and Miflin, 1974) it was generally thought that glutamate dehydrogenase (GDH) was the enzyme responsible for the incorporation of nitrogen into the α-amino group of amino acids. The reaction catalysed by GDH is as follows:

$$\text{2-oxoglutarate} + NH_3 + \text{NAD(P)H} + H^+ \longrightarrow \text{L-glutamate} + \text{NAD(P)} + H_2O$$

At the present time, however, the role of GDH in higher plant ammonium assimilation is regarded as being a minor one (Miflin and Lea, 1976; Lewis, James and Hewitt, 1982). This interpretation is based, to a large extent, on the low affinity of the enzyme for ammonium. Lee and Stewart (1978) made a comparison of the affinity of GS and GDH for ammonium acetate. In the case of GS the K_m values (the K_m being equal to the substrate concentration at which the enzyme-catalysed reaction proceeds at half its maximum rate) for this substrate were in the range 1-3 x 10^{-5} M. Those for GDH lay in the range 1-4 x 10^{-2} M. This thousand-fold difference in K_m between the two enzymes represents a considerable difference in affinity for ammonium. High levels of GDH, however, are present in a number of species suggesting that it must be of some importance in these plants. Lee and Stewart (1978) have suggested that the levels of the enzyme that are being observed represent "de-repressed" levels, and as such provide a means of compensating for the enzyme's low affinity for ammonium. Alternatively, these workers point out that GDH could, under conditions where photosyntheses and hence energy supply is limited, circumvent the energy requirements of the GS-GOGAT pathway by providing a route for the direct assimilation of ammonium into glutamate. Consequently, studies which examine the behaviour of ammonium assimilating enzymes in plants under stress conditions may prove useful in elucidating their respective rôles in nitrogen assimilation.

In plant roots, however, energy is supplied by respiration. Lewis _et al_. (1983) suggest that the function of GDH could be to maintain an adequate level of oxoglutarate in the root. That is, in higher plants GDH functions in the opposite role to that originally ascribed to it, being responsible for the production of 2-oxoglutarate from glutamate and not vice-versa. This would enable the tricarboxylic acid cycle to operate even when nitrogen assimilation, involving the GOGAT reaction with its high utilisation of oxoglutarate, was rapid.

2.5 Ammonium Plus Nitrate as a Nitrogen Source

A mixture of ammonium and nitrate as a nitrogen source, rather than either ion supplied alone, has been observed to produce greatest growth and protein production in many plant species.

2.5.1 Mixed Nitrogen Nutrition and Plant Growth

Weight gains by introducing ammonium into an all nitrate nutrient medium have been observed in species as diverse as soybean tissue culture and flax (Gamborg and Shyluk, 1970), wheat (Gamborg and Shyluk, 1970; Cox and Reisenauer, 1973), *Dianthus caryophyllus* L. (Green *et al*., 1973) and *Zea mays* (Warncke and Barber, 1973; Domska, 1974). Cox and Reisenauer (1973) suggested that the yield and growth enhancement that they observed in wheat (*Triticum aestivum* L. cv. "INIA") was a result of the reduced energy requirement in using ammonium instead of nitrate during protein synthesis, and from increased photosynthetic capacity.

The influence of ammonium and nitrate nitrogen on the growth of *Zea mays* was investigated by Schrader *et al*., 1972. A concentration of 100 ppm of nitrogen was supplied at the beginning of the experiment and samples were harvested for analysis at weekly intervals over a four week period. Fresh weights of plant parts were generally higher when the plants were supplied their nitrogen as a mixture of ammonium and nitrate (nitrate: ammonium ratios of 25:75, 50:50 and 75:25) than when either nitrate or ammonium were provided alone. Shoot:root ratios increased from an average of 1.4 at the first sampling to 3.5 at the fourth, and final, sampling.

Overall, the total fresh weight harvested in four samplings was highest for the 50:50 treatment, and lowest for treatments with only nitrate as the nitrogen source. Dry weights followed the same trends as those for fresh weights.

In a study of the effect of ammonium:nitrate ratio in *Zea mays* by Warncke and Barber (1973), it was reported that the lowest production of dry matter was obtained at the high ammonium:nitrate ratio of 8.4. This was probably related to the onset of ammonium toxicity. Haynes and Goh (1977) found that *Callistephus chinensis* could tolerate higher levels of ammonium when levels of nitrate in the growth media were also high. Such levels of ammonium would have normally been toxic.

2.5.2 Total Nitrogen Concentration in Plant Tissues

The concentration of total nitrogen in plant parts was found to be greater when ammonium or ammonium plus nitrate was supplied rather than nitrate alone (Cox and Reisenauer, 1973). These workers also pointed out that the total nitrogen content of plant tissues was not a reliable index of the nitrogen status of plants supplied with ammonium or ammonium plus nitrate, since this value continued to increase as yields were reduced as a result of ammonium toxicity.

In roots of *Zea mays*, Schrader *et al*. (1972) reported that the highest total nitrogen levels were found in roots supplied with ammonium only. Nitrate:ammonium ratios of 1:1 and 1:3 produced higher total nitrogen levels than when plants were fed nitrate only. It was suggested that because the roots of maize plants grown on ammonium were higher in total nitrogen than those on nitrate, ammonium was detoxified in roots through direct incorporation into organic nitrogen, whereas nitrate was transported to the leaves for enzymic reduction by nitrate reductase.

2.5.3 Mixed Nitrogen Nutrition and Protein Production

It is well documented that protein production in plants is higher when a combination of ammonium and nitrate is supplied rather than either of these ions alone (Delwiche, 1951; Gilmore, 1953; Weissman, 1964; Domska, 1974). Weissman (1964) reported that dry weight, total protein, protein per unit weight and protein as a percentage of total nitrogen were all greater in leaves of sunflower (H. *annuus*) fed on ammonium plus nitrate.

From amino acid analysis of leaf blade tissue, Schrader *et al.* (1972) found that percent protein in maize was highest when nitrate and ammonium were supplied in a 1:1 ratio (26%) and lowest in the nitrate only treatment (15.6%).

The quality, as well as quantity, of leaf protein has also been shown to vary with nitrogen source. Both Domska (1974) and Schrader *et al.* (1972), working on *Zea mays*, observed that the nutritive value of protein was decreased with nitrate as the sole nitrogen source as a result of a reduction in methionine content. Ammonium alone increased the aspartate content of leaves.

CHAPTER 3

MATERIALS and METHODS

3.1 ^{15} Feeding Experiments

3.2 Plant Material

Barley plants were grown in five litre plastic pots fitted with plastic gauze lids and perspex pot dividers to provide duplicate batches of plant material. Seeds of *Hordeum vulgare* L.cv. Clipper (S.A. Malsters (Pty) Ltd., Caledon, Cape Province, South Africa) were germinated on the gauze suspended over the water-filled pots. Plant material for all the experiments was grown for 20 days in a Conviron growth cabinet in a controlled environment of a 15h day (photon flux rate of 300μE $m^{-2}s^{-1}$ provided by Sylvania (GTE Sylvania Canada Ltd., Drummondville, Quebec, Canada) cool-white fluorescent lamps and 60W incandescent lamps), a day temperature of 20°C and a night temperature of 15°C with a relative humidity of 75%. On the seventh day the water was replaced by nitrogen-free Long Ashton nutrient medium (Hewitt, 1966) as shown in Tables 1 and 2, with the appropriate nitrogen supplement added.
The supplements used were:
a) 2mM KNO_3; b) 2mM NH_4Cl; c) 1mM KNO_3 + 1mM NH_4Cl or
d) 1.5mM KNO_3 + 0.5mM NH_4Cl.
Nutrient solutions were replaced every 48h up to day 14 and then changed daily until harvesting at day 20. The nutrient solutions were vigorously aerated using a small compressor connected via a perforated polyethylene

manifold to prevent the excretion of nitrite observed in young barley plants in oxygen deficient conditions (Lee, 1979; Lewis, Watson and Hewitt, 1982). To prevent ammonium toxicity (Hewitt, 1966; Maynard and Barker, 1969), 1g of calcium carbonate per millimole of ammonium was added to each litre of nutrient solution in treatments using ammonium as a nitrogen source. The feeding solution pH was monitored daily and remained in the range pH 6.0 to 6.5.

Table 1.

Macronutrients, in g 10ℓ^{-1}, used to make up nutrient solutions at the four feeding levels, each having a total nitrogen concentration of 2mM.

Macronutrient	2mM nitrate	2mM ammonium	1mM nitrate + 1mM ammonium	1.5mM nitrate + 0.5mM ammonium
$Na_2HPO_4.12H_2O$	1.42	1.42	1.42	1.42
$MgSO_4.7H_2O$	2.48	2.48	2.48	2.48
$CaCl_2.2H_2O$	3.36	3.36	3.36	3.36
K_2SO_4	1.58	1.58	1.58	1.58
KNO_3	2.02	----	1.01	1.52
NH_4Cl	----	1.07	0.54	0.27

Table 2.

Micronutrients, in mg 10ℓ^{-1}, used to make up nutrient solutions. The levels of micronutrients were the same for all feeding regimes.

Micronutrient	Mass
HBO_3	85.8
$MnSO_4.4H_2O$	46.4
$ZnSO_4.7H_2O$	6.6
$CuSO_4.5H_2O$	2.4
$Na_2MoO_4.2H_2O$	0.6
$[CH_2N(CH_2COO)_2]_2FeNa_2$ (FeEDTA)	330.0

Both Tables from Hewitt, 1966.

3.3 Isotope Feeding

The evening before the commencement of a ^{15}N feeding experiment the ^{14}N nutrient solution in each pot was renewed and the ^{15}N was prepared and placed in the growth cabinet overnight to equilibrate. Two hours after the beginning of the light period the following morning the ^{14}N nutrient solutions were replaced by nutrient solutions containing 2mM 99A%E ^{15}N KNO_3, 2mM 99A%E ^{15}N NH_4Cl (BOC-Prochem, Deer Park Road, London, U.K.), a 2mM 1:1 mixture of both ^{15}N sources, or a 1.5mM ^{15}N KNO_3 + 0.5mM ^{15}N NH_4Cl mixture. After removing the plant material from the ^{14}N feeding solution the roots were thoroughly washed with de-ionised water before being immersed in the ^{15}N feeding solution.

3.4 Harvesting and Extraction

Duplicate batches of plant material were harvested 4 and 8 hours after the start of the ^{15}N feeding experiment. Immediately after removing the plants from the feeding solution the roots were washed in de-ionised water and blotted dry. The plants were then quickly divided into root, shoot base + seed remnant and shoot. The total fresh weight of each plant part and the total number of plants was recorded for each duplicate batch. The tissue from each plant part was then coarsely chopped and mixed and 10g fresh weight was killed in liquid nitrogen before being homogenised in cold 80% ethanol (1g fresh weight of tissue per 25ml of ethanol). Extraction was then continued for 24h in a cold room at 0°C and the homogenate occasionally shaken to re-suspend the plant tissue and aid the extraction process. Figure 1 is a flow chart depicting the procedure followed during the separation of the plant extract into nitrogen fractions.

3.5 Separation and Quantification of Nitrogen Fractions

At the end of the extraction period the homogenate was filtered using a Buchner apparatus and Whatman No.1 filter paper. The residue (the bound nitrogen fraction) was washed with de-ionised water and the washings added to the filtrate containing the soluble nitrogen fraction. This alcoholic extract was blown down under an airstream to a volume of 20ml and then re-filtered, if necessary, with the precipitate being added to the original residue. The re-filtered extract and washings were again reduced in volume to 20ml. To remove chlorophyll and lipid material the extracts were shaken with ± 10ml petroleum ether. The samples were then frozen and the petroleum ether decanted off.

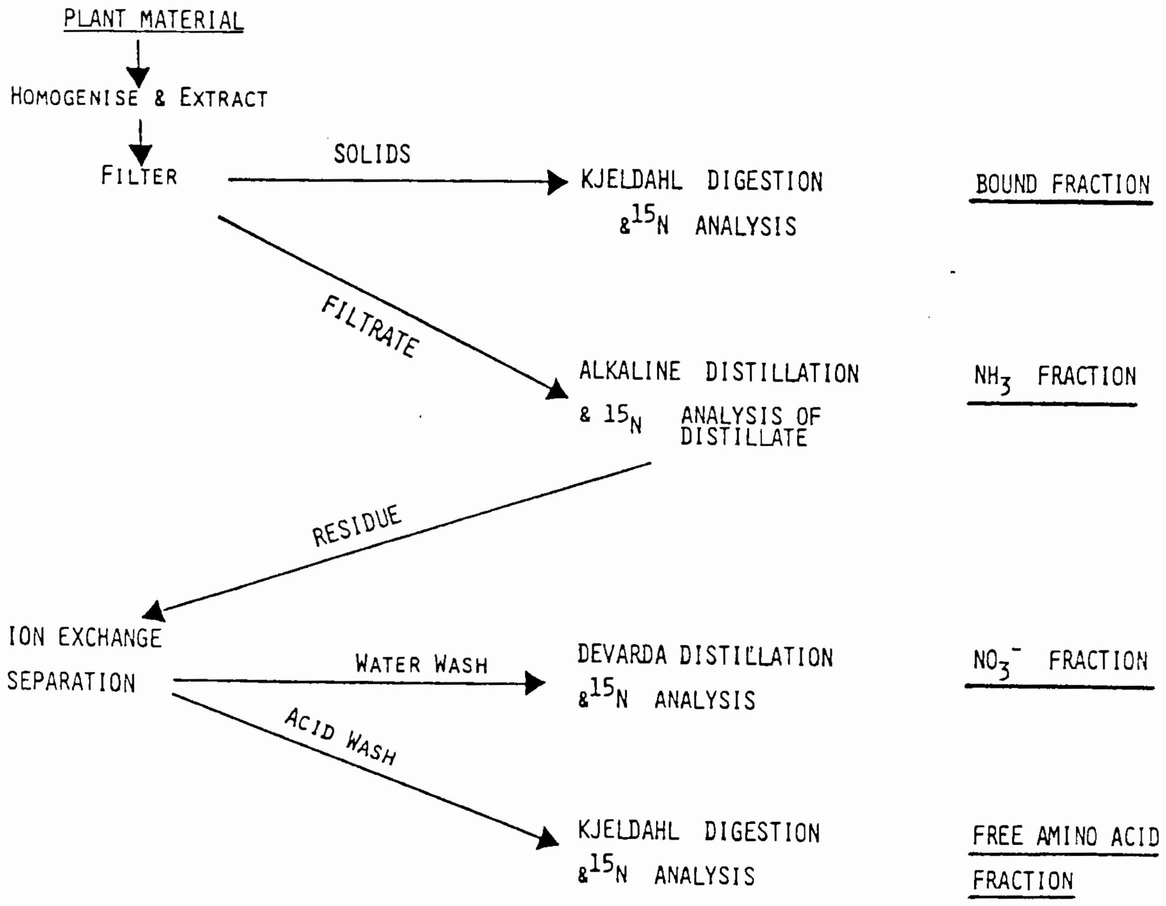

Figure 1

The preparation of plant extracts for ^{15}N determinations after feeding with nutrient solutions containing labelled nitrogen as either 2mM nitrate, 2mM ammonium, or nitrate and ammonium in a 2mM 1:1 or 2mM 3:1 mixture.

3.5.1. Treatment of the Bound Nitrogen Fraction

The filtered residue was placed in an oven at 80°C for 24h and dried to constant weight. Samples of 0.15g (with each sample taken in duplicate) were digested in a Kjeldahl flask with 3ml of nitrogen-free concentrated sulphuric acid and a 1g sodium sulphate-mercuric chloride catalyst tablet (B.D.H., Poole, Dorset, U.K.). The resulting digest was made up to a volume of 50ml with de-ionised water and 10ml aliquots taken for distillation. The ammonia present was distilled off in a Markham semi-micro distillation unit after alkalising with 15ml of 50% sodium hydroxide + 2.5% sodium thiosulphate (w/v). Approximately 30ml of the distillate was collected in 2ml of 0.02N HCl and the amount of nitrogen present was determined by back titration with 0.005N NaOH using Tashiro's indicator (screened methyl red). After titration, the sample was re-acidified with approximately 0.1N HCl to prevent the loss of ammonia. The distillate was then blown down under an airstream to a suitable volume for ^{15}N determination, the optimum amount of nitrogen being 25μg per 0.2ml of sample.

3.5.2. Treatment of the Soluble Nitrogen Fraction

To separate the nitrate and organic + ammonium nitrogen fractions the filtrate was passed through a 3 x 1 cm Dowex 50W-X8 standard H^+ (100-200 mesh particle size) ion exchange column (Atkins and Canvin, 1971). The water-soluble nitrate fraction was eluted with 50ml of de-ionised water and the remaining organic + ammonium fraction with 100ml 2N HCl. This procedure was necessary in all but the 2mM NH_4^+ feeding experiment.

3.5.2.1 Treatment of the Nitrate Fraction

The eluate containing the nitrate fraction was reduced in volume under an airstream to 20ml, and duplicate 8ml aliquots were placed in a Markham semi-micro distillation unit along with 0.3g of Devarda's Alloy as a reductant and 0.2g A.R. magnesium oxide which had previously been heated to 600°C in a muffle furnace for 2h in order to drive off CO_2 which otherwise may have interfered with the titration. During the steam distillation the first 30ml of the ammonia distillate was collected in 2ml of 0.02N HCl. The titration procedure and the preparation of the sample for ^{15}N analysis was carried out as outlined for the bound nitrogen fraction (Section 3.5.1).

3.5.2.2 Treatment of the Ammonium + Organic Nitrogen Fraction

The eluate containing the ammonium + organic nitrogen fraction was evaporated under an airstream to 20ml. Duplicate 8ml aliquots were placed in a Markham semi-micro distillation unit with 0.2g A.R. magnesium oxide and the ammonium fraction distilled over. From distillations carried out on samples containing known concentrations of asparagine and glutamine it was shown that if this mild alkali distillation was restricted to 3-4 minutes duration then no hydrolysis of the amides occurred and only the ammonium nitrogen would be released. The distillate was collected, titrated and prepared for ^{15}N analysis as described previously. The residue from this distillation, containing the soluble organic nitrogen, was allowed to cool and then filtered to remove the magnesium oxide before being reduced in volume (to ± 10ml), and transferred to a Kjeldahl flask. After adding

3ml of concentrated, nitrogen-free sulphuric acid and a 1g sodium sulphate-mercuric chloride catalyst tablet the fraction was digested. The resulting digest was made up to 20ml with de-ionised water and duplicate 8ml aliquots taken for distillation. The steam distillation, titration and preparation of the sample for ^{15}N analysis were as described for the bound fraction.

3.6 ^{15}N Analysis

3.6.1 Sample Preparation

The acidified samples were prepared for ^{15}N analysis by molecular emission spectroscopy (Statron NOI-4 molecular emission spectrophotometer) according to the sodium hypobromite method of Faust (1967). Using an alkaline hypobromite solution as the oxidant, the sample was reacted under vacuum to release nitrogen gas:

$$2NH_3 + 3NaOBr \text{ ------> } N_2 + 3H_2O + 3NaBr$$

The vacuum system consisted of a high vacuum pump, which created a pre-vacuum of 0.1kPa, connected in series with a mercury diffusion pump giving the system a final pressure of 1.0Pa. Two liquid nitrogen cold traps were used to reduce the water vapour pressure of the system.

3.6.2 Calculation and Expression of ^{15}N Results

The discharge tubes containing nitrogen gas released from the samples were placed between the terminals of an RF oscillator in the Statron molecular emission spectrophotometer, and the gas excited. A red/violet colour gave optimum results with a blue/white colouration indicating the presence of water vapour or bromine, the latter being produced during the final oxidation step in the reaction between an over-acidified sample and sodium hypobromite. On exciting the nitrogen gas the three isotopic molecules $^{15}N^{15}N$, $^{15}N^{14}N$ and $^{14}N^{14}N$ each emit a characteristic bandhead which is photoelectrically recorded. A typical trace showing the characteristic "fingerprint" of each peak for ^{15}N enrichments below 50% is shown in Figure 2. The percentage enrichment was calculated using the formula:

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

where A and B are the bandheads of the $^{14}N^{14}N$ and $^{14}N^{15}N$ molecules respectively, and Va and Vb are the gain settings on the molecular emission spectrophotometer at which the bandheads A and B were recorded.

For ^{15}N enrichments above 50% a typical trace for the three peaks is shown in Figure 3. To calculate the % En for ^{15}N enrichments above 50% a modification of the above formula was used:

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

All ^{15}N enrichment figures obtained were averages of three complete traces. A calibration curve (Figure 4) was drawn up for the Statron and was used to correct the enrichment figures. In order to obtain the %En in excess of the natural abundance (the atom percent excess, A%E), the natural abundance (0.37%) was subtracted from the corrected %En. The ^{15}N content of a particular sample, expressed as micrograms ^{15}N per gram fresh weight (μg ^{15}N g^{-1} fw), was calculated by multiplying the total nitrogen content (obtained from the distillation procedure) by the A%E value. This value was then multiplied by the average weight of the plant part (root, shoot + base + seed remnant or shoot) represented by the sample, thus giving the results finally as micrograms ^{15}N per plant part.

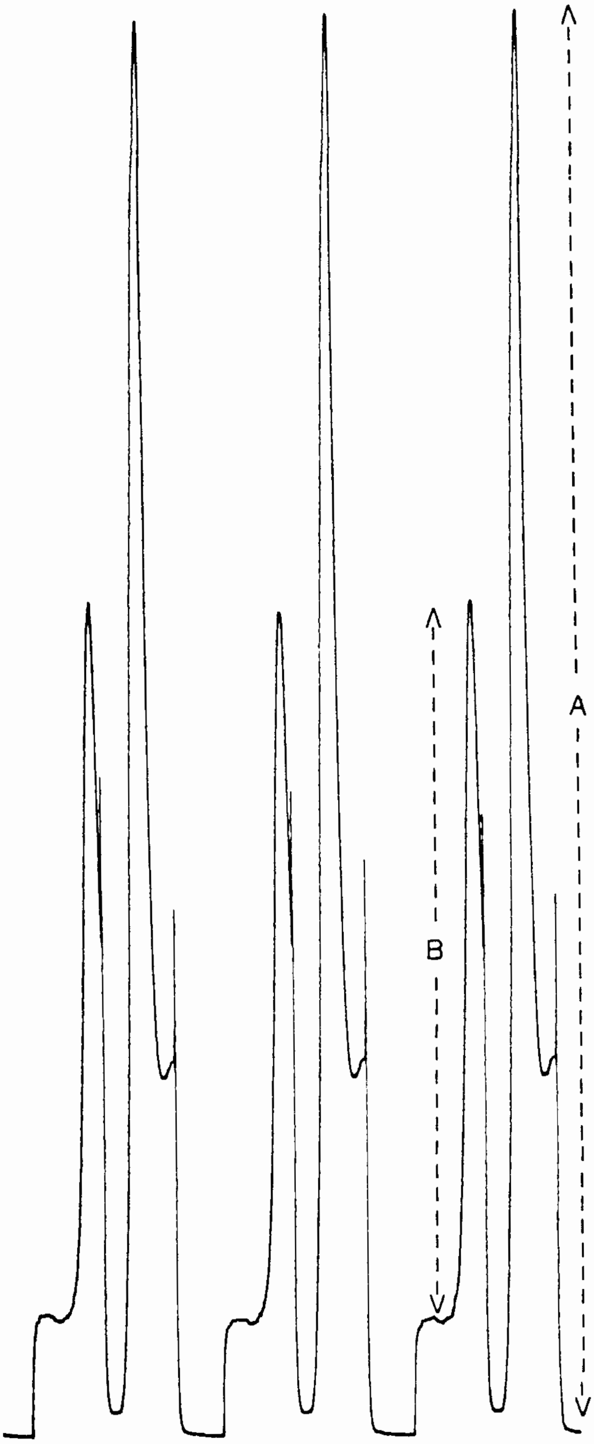

Figure 2

Typical traces for ^{15}N enrichments below 50%, showing good separation of the nitrogen molecules $^{14}N\,^{15}N$ and $^{14}N\,^{14}N$ where A and B represent the peak heights of the $^{14}N\,^{14}N$ and $^{14}N\,^{15}N$ bandheads respectively.

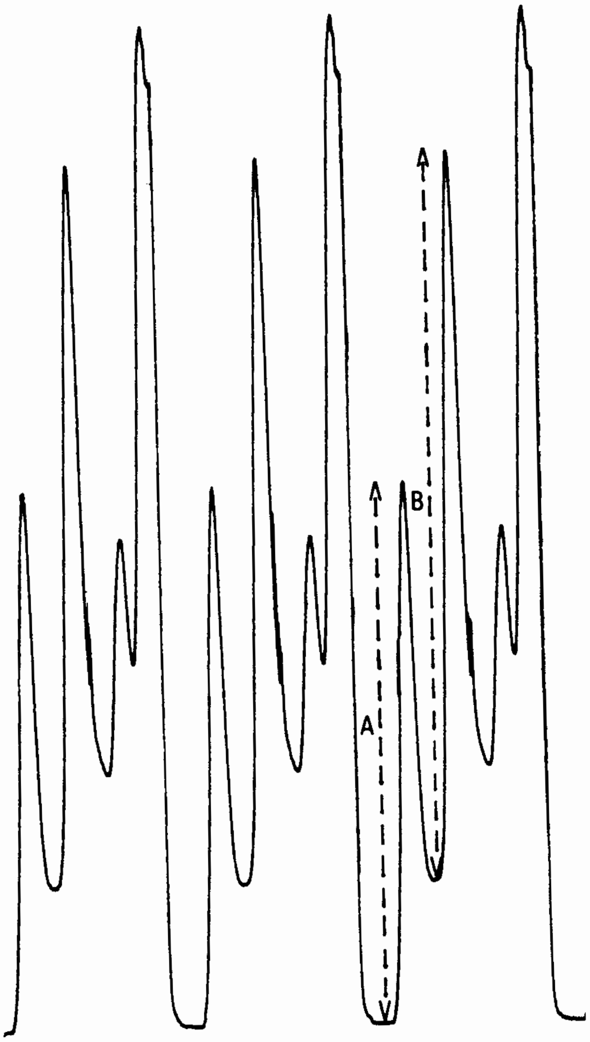

Figure 3

Typical traces for ^{15}N enrichments above 50%, showing good separation of the nitrogen molecules $^{15}N\,^{15}N$ and $^{14}N\,^{15}N$ where A and B represent the peak heights of the $^{15}N\,^{15}N$ and $^{14}N\,^{15}N$ bandheads respectively.

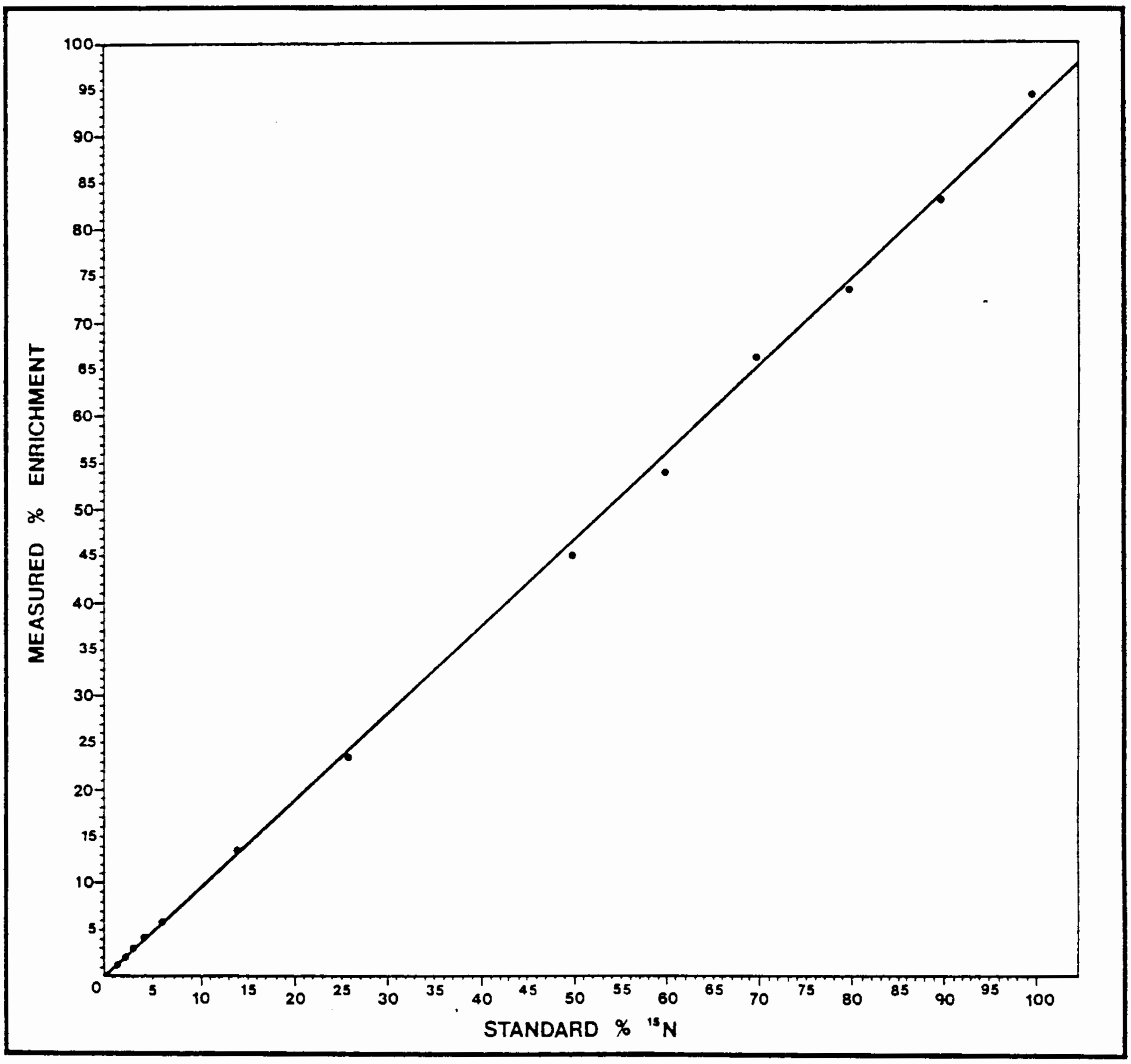

Figure 4

Standard curve for the correction of ^{15}N enrichments determined with the Statron molecular emission spectrometer.

3.7 Xylem Sap Analysis

3.7.1 Collection of Xylem Sap

Xylem sap was collected during the course of ^{15}N feeding experiments from plants which were not harvested for analysis. The shoots of approximately 30 plants were severed just below the insertion of the first leaf. The stumps were washed with de-ionised water and blotted dry. Sap exuding from the cut surface was collected using a Pasteur pipette for one hour, the collection period beginning half an hour before the harvesting of the plants to be used for ^{15}N analysis, and continuing for a further half hour after harvesting. The test tubes containing the collected sap were kept in an ice-box throughout the collection period and then placed in a freezer until needed for analysis.

3.7.2 Separation of the Nitrogen Fractions

The nitrate and organic + ammonium nitrogen fractions were separated using an ion exchange column as described in Section 3.5.2.

3.7.2.1 Treatment of the Nitrate Fraction

Nitrate determinations were made by the distillation method, as described in Section 3.5.2.1 and also using Szechrome NAS reagent (diphenylamine sulphonic acid chromogene, obtained from Yedetek Ltd., Rimon 10, Omer 84065, Israel). To 0.5ml of diluted sap was added 2.5ml of Szechrome NAS reagent dissolved in a 1:1 mixture of concentrated phosphoric and sulphuric acids. After shaking, the colour was allowed to develop for 15 minutes before measuring the absorbance at 570nm on a Beckman Model 42 spectrophotometer. A series of standards were prepared using known concentrations of potassium nitrate and assayed simultaneously with the sap samples. A calibration curve (Figure 5) was plotted allowing the amount of nitrogen present as nitrate in the xylem sap to be estimated.

3.7.2.2 Treatment of the Organic + Ammonium Nitrogen Fraction

This fraction received the same treatment as the comparable fraction obtained from the homogenised plant material as described in Section 3.5.2.2.

3.7.3 The Separation and Quantitative Determination of Amino Acids

The amino acid composition of the xylem sap was determined using a Beckman 120C amino acid analyser (Beckman Instruments, INC., Fullerton, CA 9234, U.S.A.). To separate the amino acids ± 200µl of the sample was loaded onto a 22cm column of Beckman UR 30 spherical ion-exchange resin. Three lithium citrate buffers, buffer A, B and C, were employed in sequence (Kedenburg, 1971), with pH's of 2.83, 3.70 and 3.75 respectively. The separated components were automatically mixed with ninhydrin and the reaction mixture passed through a hot water bath to allow the characteristic purple/blue colour to develop. An internal standard was used to determine the decay of the ninhydrin with time which allowed a correction factor to be applied to the amino acid determinations. The optical density was measured at 540nm and recorded on a Beckman 125 digital integrator and a 3 channel chart recorder. From the optical density and a specific conversion constant for each amino acid a quantitative value for each amino acid was calculated.

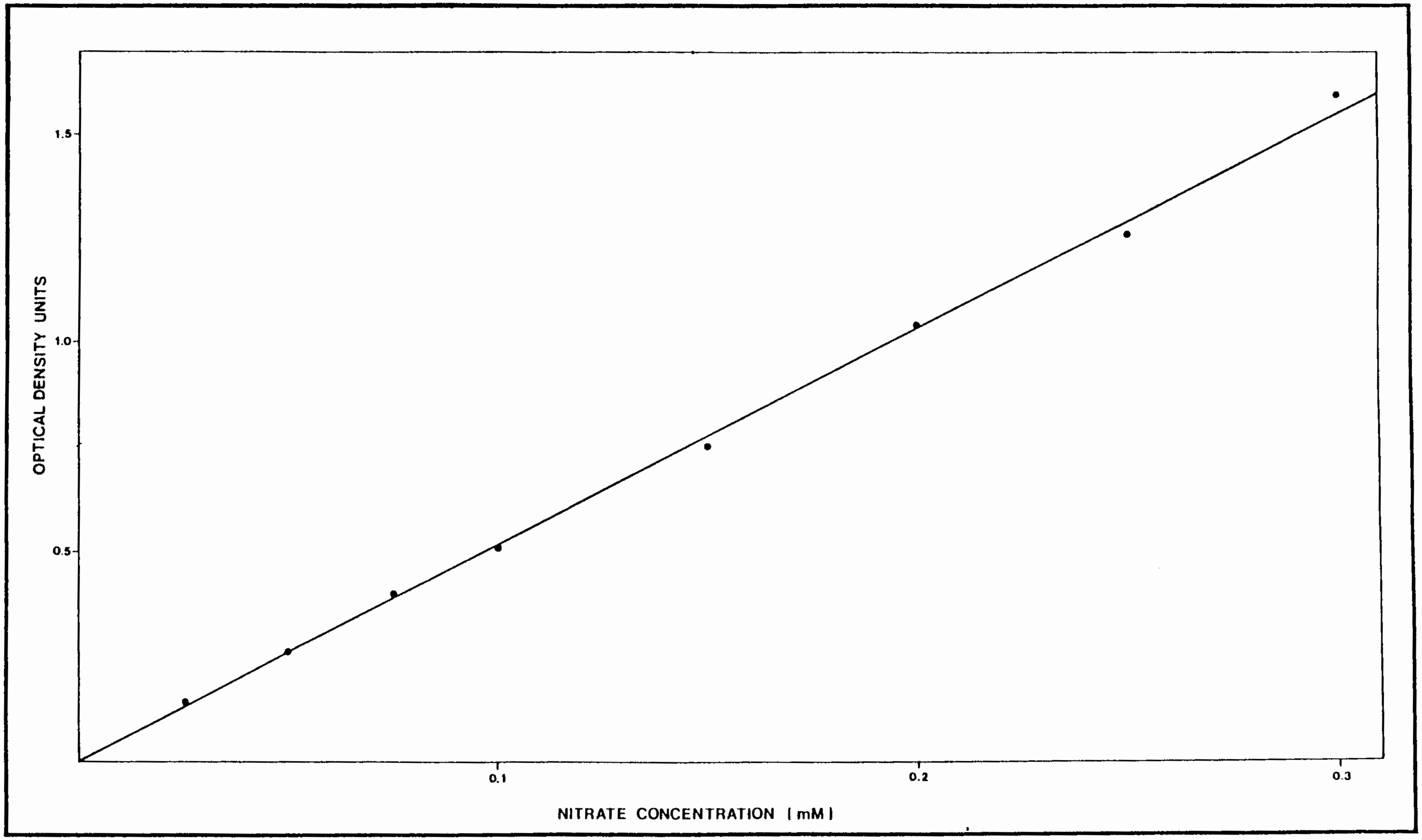

Figure 5
Standard curve for the colorimetric determination of nitrate concentration using Szechrome NAS reagent.

3.8 Feeding of ^{15}N-Glutamine to Methionine Sulfoximine-Treated roots

3.8.1 Plant Material

Two batches of approximately 100 barley plants (each divided into duplicate samples of 50 plants) were grown following the technique and environmental conditions described in Section 3.2. The nitrogen supplement used was 2mM ammonium chloride.

3.8.2 Feeding of ^{15}N-Glutamine plus MSO to Roots

On the morning of the experiment the plants were placed in fresh nutrient solution containing 2mM NH_4Cl and 5mM L-methionine-DL-sulfoximine (Sigma Chemical Co., London, U.K.), for 4 hours. This time period was long enough to allow the MSO (a glutamine synthetase inhibitor) to inactivate the enzyme. At the end of the 4 hour period the solution was again replaced by fresh nutrient solution containing 2mM ammonium chloride, 5mM MSO and 2mM ^{15}N-glutamine.

3.8.3 Harvesting and Extraction

Batches of plants were harvested 2 and 4 hours after feeding with ^{15}N-glutamine. The roots were washed in de-ionised water, blotted dry and 10g fresh weight from each duplicate set of plants homogenised in cold 80% ethanol and allowed to extract for 24 hours in a cold room at 0°C. The homogenate was then filtered and the filtrate containing the soluble nitrogen fraction retained for amino acid analysis. The solid residue was discarded.

3.8.4 Treatment of the Soluble Nitrogen Fraction

The soluble nitrogen fraction was blown down under an airstream to a volume of 20ml and approximately 100μl taken for amino acid analysis. The sample was loaded onto a 1.25 metre x 3cm column of Beckman UR 30 spherical ion-exchange resin. The eluent used was a lithium citrate buffer at pH 2.83. As the eluate containing the individual amino acids left the column it was collected in the test tubes of a fraction collector. One microlitre was taken from each tube in sequence and spotted onto chromatography paper which was then sprayed with ninhydrin and dried in an oven for 10 minutes at 90°C. The test tubes containing the bulk of each amino acid could be isolated by reference to the intensity of the colour reaction in the spot test. The only amino acids that were present in sufficient quantity for ^{15}N determinations were glutamate and glutamine, threonine, serine, alanine, glycine, aspartate and asparagine. To verify the identification of the amino acids some of the eluate from this column was channelled into a smaller column, 9cm x 1.2cm, and eluted with sodium citrate buffer at pH 5.25 (0.35N). The eluate containing the separated amino acids from the short column was automatically mixed with ninhydrin and passed through a hot water bath for colour development. The optical density was measured at 540nm as the mixture flowed through a photocell and recorded on a Beckman 125 digital integrator and a 3 channel chart recorder.

Each amino acid solution was reduced in volume under an airstream to approximately 10ml and put through the Kjeldahl digestion and distillation procedure (outlined in Section 3.5.2.2) before undergoing ^{15}N analysis (Section 3.6).

3.9 ^{15}N-ammonium Feeding Procedure

3.9.1 Plant Material

Two batches of approximately 100 barley plants (each divided into duplicates of 50 plants) were grown following the technique and environmental conditions described in Section 3.2.

3.9.2 ^{15}N Feeding Procedure

Duplicate batches of 50 plants were removed for extraction after a 30 minute and a 120 minute feeding period. The feeding procedure was the same as that described in section 3.3, with the 2mM ^{14}N-ammonium nutrient solution being replaced by 2mM ^{15}N-ammonium feeding solution just prior to the beginning of the experiment.

3.9.3 ^{15}N-ammonium plus MSO Feeding Procedure

The feeding procedure was carried out as described above and in Section 3.3 with the following exception. In order to achieve complete inhibition of glutamine synthetase before the commencement of ^{15}N-ammonium feeding, MSO was added to the ^{14}N-ammonium feeding solution at a concentration of 5mM. This was done 4 hours before this solution was replaced by the ^{15}N-ammonium feeding solution, which also contained 5mM MSO.

3.9.4 Harvesting and Extraction of Roots

Batches of plants were harvested 30 and 120 minutes after commencement of the feeding experiment. The harvesting process and extraction procedure were carried out as described in Section 3.8.3.

3.9.5 Analysis and Collection of Free Amino Compounds

The isolation and collection of the free amino compounds in sufficient quantities for ^{15}N analysis was performed on a 150cm preparative column of M84 ion exchange resin (25µm particle size), and a 12cm column of PA35 resin (13µm particle size) (Beckman Instruments) for the collection of ammonia. The eluates were collected in a fraction collector.

3.9.6 ^{15}N Analysis

The column-eluate fractions containing amino compounds were reduced in volume to approximately 10ml and then subjected to Kjeldahl digestion and alkaline distillation as outlined in Section 3.5.2.2. The ^{15}N enrichments of the resulting samples were determined by the method described in Sections 3.6.1 and 3.6.2.

3.10 Six Week Growth Experiment: to Determine Fresh and Dry Weight and Total Nitrogen Content of Mature Barley Plants.

3.10.1 Plant Material

The barley plants were grown according to the technique and environmental conditions outlined in Section 3.2. The plant material was grown for 6 weeks and two pots of barley plants (with each pot divided to provide duplicate batches) were raised per nitrogen regime thus providing quadruplicate results. The nitrogen supplements used were 2mM potassium nitrate, 2mM ammonium chloride, and 1mM potassium nitrate plus 1mM ammonium chloride.

3.10.2 Harvesting

Quadruplicate batches of plant material were harvested at the end of 6 weeks growth. Immediately after removing the plants from the feeding solutions the roots were washed with de-ionised water and blotted dry. The plants were then divided into root and shoot (plus seed remnant). The total number of plants and the total fresh weight of root and shoot tissues in each replicate batch were recorded. All the tissue from each batch was then placed in individual aluminium foil trays and dried in an oven at 80°C to constant weight (approximately 24 hours). The dry weight of each batch of root and shoot was recorded.

3.10.3 Total Nitrogen Analysis

The dried and weighed plant material was ground in liquid nitrogen and 0.15g samples of the resulting powder were weighed out for analysis. The procedures for the Kjeldahl digestion and the alkaline distillation were as described in Section 3.5.1.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 General Observations

From a visual comparison of plants fed with each of the four different nitrogen sources, those from the mixed (nitrate plus ammonium) nitrogen feeding treatments were generally larger and more robust than those fed on a single nitrogen source. In Tables 3.1a,b,c and d, average masses of individual 20 day-old barley seedlings from each of the feeding regimes are shown along with their total nitrogen and organic nitrogen contents. The 3:1 nitrate : ammonium mixed feed plants were the largest of all and had the greatest total mass, being on average, 1.5 times heavier than 1:1 mixed feed feed plants, 1.7 times heavier than the nitrate-only fed plants and 2.1 times heavier than ammonium-fed plants. The highest rate of incorporation of ^{15}N into organic nitrogen, however, was exhibited by the 1:1 mixed feed plants, followed by the ammonium-fed plants, the 3:1 nitrate : ammonium mixed feed plants and nitrate-fed plants, (see Sections 4.2.1., 4.2.2., and 4.2.3.).

Apart from the variation in size there were other differences in the appearance of plants according to the nitrogen source supplied. The nitrate plants had very erect, fibrous stems with the leaves a lighter shade of green than plants from the other treatments. The root system was dense and extensive. The shoots of ammonium plants were much softer with a tendency to lodge and the leaves were a dark green colour. Although the pH of the nutrient solution was monitored and controlled (Section 3.2), a retardation in the growth of the roots was apparent, as previously reported by De Kock and Kirkby (1969) and Cox and Reisenauer (1973). The root system formed a tight ball and the root surfaces were covered with many nodule-like protuberances. The plants from the mixed feed treatments differed from each other only in size. They were robust plants, taller than those from other treatments, with extensive root systems.

The reasons for the differences in appearance between plants from the four feeding regimes are discussed in Chapter 5, Section 5.1.

Table 3.1

Mass and nitrogen content of whole plant, shoot, root and shoot base + seed regions of individual 20-day-old barley plants fed separately with 2mM nitrate (a), or 2mM ammonium (b).

	(a) 2mM NO_3^-			(b) 2mM NH_4^-		
	Total Mass (g)	Total N (mg)	Organic N (mg)	Total Mass (g)	Total N (mg)	Organic N (mg)
WHOLE PLANT	0.884	2.388	2.145	0.726	2.917	2.863
SHOOT	0.410	1.582	1.471	0.367	2.042	2.025
SHOOT BASE + SEED	0.172	0.306	0.261	0.095	0.204	0.202
ROOT	0.300	0.500	0.413	0.264	0.671	0.636

Mean of 160 plants used for each feeding treatment.

Table 3.1 (cont).

Mass and nitrogen content of whole plant, shoot, root and shoot base + seed regions of individual 20-day-old barley plants fed separately with a 1mM nitrate : 1mM ammonium mixture (c), or a 1.5mM nitrate : 0.5mM ammonium mixture (d).

	(c) 1:1 $NO_3^- + NH_4^+$			(d) 3:1 $NO_3^- + NH_4^+$		
	Total Mass (g)	Total N (mg)	Organic N (mg)	Total Mass (g)	Total N (mg)	Organic N (mg)
WHOLE PLANT	1.002	2.740	2.576	1.534	4.866	4.152
SHOOT	0.454	1.925	1.876	0.678	2.982	2.708
SHOOT BASE + SEED	0.183	0.274	0.253	0.269	0.671	0.575
ROOT	0.363	0.541	0.447	0.587	1.214	0.869

Mean of 160 plants used for each feeding treatment.

4.2 ^{15}N Feeding Experiments and Xylem Sap Analyses

4.2.1 Nitrate-fed Plants

From an examination of Table 4 it can be seen that the ^{15}N enrichments of the root, shoot and shoot base + seed in these plants were generally low when compared to the ^{15}N enrichment figures in the other three feeding regimes (Tables 6, 7, and 8). The enrichment values in these nitrate-fed plants ranged from 0.4 A%E in the bound nitrogen fraction of the shoot and shoot base + seed regions at 4h, to 23.1 A%E in the ammonium fraction of the root at 8h. With only one exception (the ^{15}N ammonium enrichment of the root) the ^{15}N enrichments of all the nitrogen fractions (nitrate, ammonium, soluble nitrogen and bound nitrogen) at least doubled between the 4h and 8h harvest, suggesting that none of the nitrogen pools were saturated, even at the 8h harvest. This slow uptake of the nitrate ion, relative to the uptake of the ammonium ion, has been observed in other species such as perennial rye-grass (Lycklama, 1963), and tobacco (De Kock, 1970).

In nitrate-only fed plants the major portion of the absorbed ^{15}N was located in the shoot. At the 4h harvest the shoot ^{15}N content was almost 3 times that of the root in the soluble organic nitrogen fraction, more than 1.5 times that of the root in the bound nitrogen fraction, and almost 1.5 times that of the root in the nitrate fraction (Table 4, Figure 6.1b). This indicates that in 20-day-old barley plants the shoot is the main organ of nitrate assimilation. Xylem sap analysis (Table 5) shows that approximately 66% of the ^{15}N supplied to the shoot via the xylem stream was in the form of nitrate with the remainder entering the shoot as organic nitrogen, mainly glutamine. From amino acid analysis it was found that, on average, 49.4% of the total organic nitrogen transported in the xylem stream was in the form of glutamine (Table 14). Asparagine was the second most abundant amino compound.

From a comparison of the ^{15}N-nitrate enrichments of the root at 4h (4.6 A%E, Table 4) and 8h (12.7 A%E), and the xylem sap at 4h (52 A%E, Table 15) and 8h (60 A%E) it can be deduced that nitrate, in these nitrate-only fed plants, must bypass any storage pools of the root and be directly transported to the shoot in the xylem stream.

Enzyme studies carried out on roots and leaves for nitrate reductase activity also give support to the view that the shoot is the predominant site of nitrate reduction and assimilation in young barley plants (Lewis, Watson and Hewitt, 1982). The average ^{15}N-ammonium enrichment in the root, however, was 20.3 A%E and the ^{15}N enrichment value of the soluble nitrogen fraction of the root doubled between 4h and 8h indicating that some nitrate reduction and assimilation did take place in the roots.

The absence of ^{15}N-ammonium in the xylem sap of these nitrate-fed plants (Tables 5 and 15) was anticipated as ammonium has the ability to disrupt photosynthesis at comparatively low internal concentrations, as also mentioned in Section 4.2.2.

Probably as a result of the slow uptake of the nitrate ion, there was a marked reduction in the incorporation of ^{15}N into organic nitrogen (5.6 μg ^{15}N h^{-1} per plant or 6.3 μg ^{15}N h^{-1} g^{-1} fresh weight) when compared to plants grown on the other three nitrogen sources (Figure 7). This unexpectedly low assimilation rate was checked by repeating the feeding experiment, but the results were found to be reproducible, differing by only 0.7 μg ^{15}N h^{-1} per plant.

The amount of ^{15}N in the bound form, in both the root and the shoot, was predictably greater at 8h than at 4h with the amount in the shoot exceeding that in the root at both harvests (Table 4 and Figure 6.1b). In the shoot the bound ^{15}N content increased by a factor of 3.5 between the 4h and 8h harvest, while the bound ^{15}N content of the root increased by a factor of 2.8.

At the 8h harvest the ^{15}N content of the bound fraction in both the root and shoot exceeded that of the soluble organic nitrogen fraction. The shoot and root ^{15}N concentrations at 8h in the bound fraction were 18.4 and 8.6 µg ^{15}N per plant respectively, and in the soluble organic nitrogen fraction 16.4 and 4.8 µg ^{15}N per plant.

As can be seen from Tables 3.1a,b,c and d, these nitrate-fed plants also had the lowest total organic nitrogen content of the four treatments, but from the apparently poor ^{15}N-nitrate assimilation rate, this value was not as low as would be expected. The reason for this was not immediately apparent, but at least a partial explanation would be that a large proportion of the organic nitrogen would have been supplied to the seedling by the redistribution of ^{14}N from the protein contained in their germinated seeds.

Table 4.

Concentrations and ^{15}N enrichments (A%E) of the nitrate N, ammonium N, soluble N and bound N fractions of individual shoot, root and shoot base (+ seed remnant) regions of 20-day-old barley plants fed 2mM NO_3^- and harvested 4 hours and 8 hours after commencement of ^{15}N feeding. Figures have been averaged from duplicated determinations of replicate 80 plant batches and are expressed as mean ± standard deviation.

		ROOT			SHOOT			SHOOT BASE + SEED		
N Fraction	Harvest	N Conc. (µg N/ Plant)	^{15}N **enrich.** (A%E)	^{15}N Conc. (µg/ Plant)	N Conc. (µg N/ Plant)	^{15}N **enrich.** (A%E)	^{15}N Conc. (µg/ Plant)	N Conc. (µg N/ Plant)	^{15}N **enrich.** (A%E)	^{15}N Conc. (µg/ Plant)
Nitrate	4h	86.8 ± 3.0	4.6 ± 0.0	4.0 ± 0.4	97.0 ± 6.9	5.8 ± 0.1	5.7 ± 0.6	44.6 ± 3.3	3.2 ± 0.0	1.4 ± 0.2
	8h	87.2 ± 3.9	12.7 ± 0.0	11.1 ± 1.2	125.1 ± 6.8	13.4 ± 0.1	16.7 ± 1.0	46.2 ± 3.9	6.8 ± 0.4	3.2 ± 0.6
Ammonium	4h	5.4 ± 0.0	17.4 ± 0.3	0.9 ± 0.0	6.0 ± 0.5	5.6 ± 0.7	0.3 ± 0.0	2.4 ± 0.1	6.0 ± 0.1	0.2 ± 0.0
	8h	4.1 ± 0.0	23.1 ± 0.2	0.9 ± 0.0	7.3 ± 0.3	14.1 ± 0.0	1.0 ± 0.0	2.2 ± 0.0	12.6 ± 0.0	0.3 ± 0.0
Soluble Nitrogen	4h	47.1 ± 1.6	4.8 ± 0.1	2.3 ± 0.1	112.9 ± 6.0	5.7 ± 0.5	6.4 ± 0.4	41.0 ± 1.3	2.4 ± 0.1	1.0 ± 0.1
	8h	49.5 ± 0.0	9.8 ± 0.1	4.8 ± 0.0	112.2 ± 6.3	14.6 ± 0.8	16.4 ± 0.6	19.3 ± 0.6	5.6 ± 0.0	1.1 ± 0.0
Bound Nitrogen	4h	359.9 ± 10.0	0.9 ± 0.0	3.1 ± 0.0	1390.7 ± 14.8	0.4 ± 0.0	5.3 ± 0.3	212.3 ± 2.3	0.4 ± 0.0	0.9 ± 0.0
	8h	360.1 ± 12.7	2.4 ± 0.1	8.6 ± 0.4	1313.2 ± 18.4	1.4 ± 0.0	18.4 ± 0.2	244.2 ± 2.3	0.9 ± 0.0	2.1 ± 0.0

* Plant masses are given in Table 3a.

Table 6.

Concentrations and ^{15}N enrichments (AZE) of the ammonium N, soluble N and bound N fractions of individual shoot, root and shoot base (+ seed remnant) regions of 20-day-old barley plants fed 2mM NH_4^+ and harvested 4 hours and 8 hours after commencement of ^{15}N feeding. Figures have been averaged from duplicated determinations of replicate 80 plant batches and are expressed as mean ± standard deviation.

		ROOT			SHOOT			SHOOT BASE + SEED		
N Fraction	Harvest	N Conc. (µg N/ Plant)	^{15}N enrich. (AZE)	^{15}N Conc. (µg/ Plant)	N Conc. (µg N/ Plant)	^{15}N enrich. (AZE)	^{15}N Conc. (µg/ Plant)	N Conc. (µg N/ Plant)	^{15}N enrich. (AZE)	^{15}N Conc. (µg/ Plant)
Ammonium	4h	40.6 ± 1.7	79.4 ± 1.5	32.3 ± 0.9	11.8 ± 2.6	12.0 ± 0.3	1.4 ± 0.4	2.3 ± 0.2	8.5 ± 0.4	0.2 ± 0.0
	8h	30.2 ± 1.2	70.6 ± 1.8	21.3 ± 1.3	20.4 ± 1.6	28.0 ± 0.7	5.7 ± 0.5	2.6 ± 0.3	22.6 ± 0.8	0.6 ± 0.0
Soluble Nitrogen	4h	213.2 ± 4.1	14.6 ± 0.8	30.7 ± 1.6	211.5 ± 11.9	9.4 ± 0.2	20.1 ± 0.9	28.3 ± 2.8	7.9 ± 0.1	2.3 ± 0.2
	8h	232.5 ± 5.1	17.8 ± 0.3	41.7 ± 3.3	281.8 ± 8.8	22.0 ± 0.9	62.1 ± 2.5	34.3 ± 0.9	12.7 ± 0.2	4.4 ± 0.1
Bound Nitrogen	4h	420.6 ± 4.3	2.8 ± 0.1	11.8 ± 0.4	1710.8 ± 20.0	0.8 ± 0.0	13.7 ± 0.4	177.3 ± 1.0	0.8 ± 0.0	1.4 ± 0.1
	8h	405.0 ± 5.0	7.2 ± 0.1	29.2 ± 0.5	1846.5 ± 25.5	3.1 ± 0.1	57.4 ± 1.5	163.3 ± 1.2	2.0 ± 0.0	3.3 ± 0.1

* Plant masses are given in Table 3b.

Table 7.

Concentrations and ^{15}N enrichments (A%E) of the nitrate N, ammonium N, soluble N and bound N fractions of individual shoot, root and shoot base (+ seed remnant) regions of 20-day-old barley plants fed 1mM NO_3^- : 1mM NH_4^+ mixture and harvested 4 hours and 8 hours after commencement of ^{15}N feeding. Figures have been averaged from duplicated determinations of replicate 80 plant batches and are expressed as mean ± standard deviation.

		ROOT			SHOOT			SHOOT BASE + SEED		
N Fraction	Harvest	N Conc. (µg N/ Plant)	^{15}N enrich. (A%E)	^{15}N Conc. (µg/ Plant)	N Conc. (µg N/ Plant)	^{15}N enrich. (A%E)	^{15}N Conc. (µg/ Plant)	N Conc. (µg N/* Plant)	^{15}N enrich. (A%E)	^{15}N Conc. (µg/ Plant)
Nitrate	4h	8.3 ± 0.3	14.1 ± 0.2	1.2 ± 0.0	3.3 ± 0.3	11.9 ± 0.4	0.4 ± 0.0	4.3 ± 0.4	8.7 ± 0.1	0.4 ± 0.0
	8h	19.9 ± 2.6	32.6 ± 1.0	6.5 ± 1.0	64.0 ± 7.6	22.0 ± 0.5	14.1 ± 1.3	25.0 ± 2.1	17.0 ± 0.6	4.3 ± 0.3
Ammonium	4h	73.2 ± 2.0	95.6 ± 0.8	69.9 ± 0.4	13.9 ± 0.7	23.6 ± 0.9	3.3 ± 0.0	4.0 ± 0.2	34.8 ± 0.1	1.4 ± 0.1
	8h	86.5 ± 3.5	93.2 ± 0.5	80.6 ± 0.7	16.4 ± 0.9	35.9 ± 0.5	5.9 ± 0.1	7.4 ± 0.5	34.6 ± 0.1	2.5 ± 0.0
Soluble Nitrogen	4h	56.4 ± 1.6	35.0 ± 0.5	19.7 ± 0.1	179.7 ± 5.1	21.5 ± 0.9	38.6 ± 1.5	29.6 ± 1.0	19.0 ± 0.2	5.6 ± 0.2
	8h	47.5 ± 3.5	47.3 ± 0.9	22.4 ± 1.2	212.6 ± 6.5	45.6 ± 1.8	96.2 ± 1.0	30.9 ± 2.9	33.4 ± 0.3	10.3 ± 0.1
Bound Nitrogen	4h	397.4 ± 3.2	2.5 ± 0.2	10.0 ± 0.2	1731.4 ± 30.8	1.0 ± 0.0	16.6 ± 0.6	231.1 ± 4.9	1.0 ± 0.1	2.2 ± 0.1
	8h	393.3 ± 6.2	6.2 ± 0.2	24.5 ± 1.0	1628.6 ± 29.9	3.1 ± 0.2	51.1 ± 1.0	215.2 ± 5.0	3.2 ± 0.2	6.8 ± 0.3

* Plant masses are given in Table 3c.

Table 8.

Concentrations and ^{15}N enrichments (A%E) of the nitrate N, ammonium N, soluble N and bound N fractions of individual shoot, root and shoot base (+ seed remnant) regions of 20-day-old barley plants fed 1.5mM NO_3^- : 0.5mM NH_4^+ mixture and harvested 4 hours and 8 hours after commencement of ^{15}N feeding. Figures have been averaged from duplicated determinations of replicate 80 plant batches and are expressed as mean ± standard deviation.

		ROOT			SHOOT			SHOOT BASE + SEED		
N Fraction	Harvest	N Conc. (µg N/ Plant)	^{15}N **enrich.** (A%E)	^{15}N Conc. (µg/ Plant)	N Conc. (µg N/ Plant)	^{15}N **enrich.** (A%E)	^{15}N Conc. (µg/ Plant)	N Conc. (µg N/ Plant)	^{15}N **enrich.** (A%E)	^{15}N Conc. (µg/ Plant)
Nitrate	4h	267.4 ± 1.3	12.0 ± 0.4	32.1 ± 1.3	255.0 ± 0.4	5.3 ± 0.2	13.4 ± 0.5	90.6 ± 0.5	4.6 ± 0.2	4.1 ± 0.2
	8h	320.9 ± 4.2	11.0 ± 0.2	35.3 ± 0.6	266.7 ± 1.1	12.7 ± 0.2	33.7 ± 0.6	91.5 ± 0.6	7.7 ± 0.5	7.0 ± 0.5
Ammonium	4h	70.4 ± 3.1	80.0 ± 1.6	56.1 ± 1.4	10.8 ± 0.2	19.6 ± 1.3	2.1 ± 0.1	5.5 ± 0.1	24.2 ± 0.7	1.3 ± 0.1
	8h	30.1 ± 0.7	64.5 ± 1.4	19.4 ± 0.8	11.3 ± 0.2	22.1 ± 0.8	2.5 ± 0.1	5.8 ± 0.1	21.1 ± 0.5	1.2 ± 0.0
Soluble Nitrogen	4h	75.5 ± 5.2	22.0 ± 0.1	16.6 ± 1.2	143.6 ± 5.4	12.3 ± 0.0	17.7 ± 0.7	62.7 ± 1.3	12.3 ± 0.5	7.7 ± 0.4
	8h	83.0 ± 0.3	19.0 ± 0.9	15.8 ± 0.7	238.5 ± 7.0	10.3 ± 0.2	24.6 ± 0.6	62.8 ± 1.4	15.8 ± 0.0	9.9 ± 0.2
Bound Nitrogen	4h	776.3 ± 0.7	3.0 ± 0.3	23.1 ± 2.6	2499.0 ± 34.3	1.0 ± 0.0	25.5 ± 0.1	494.7 ± 3.3	1.3 ± 0.1	6.3 ± 0.3
	8h	803.9 ± 2.4	4.6 ± 0.1	36.6 ± 1.0	2535.7 ± 19.6	2.0 ± 0.1	49.2 ± 1.1	528.8 ± 14.0	3.9 ± 0.6	20.4 ± 1.1

* Plant masses are given in Table 3d.

4.2.2 Ammonium-fed Plants

When compared to the absorption of nitrate in nitrate-fed plants the absorption of ammonium was relatively rapid in ammonium-fed plants with the free ammonium pools of the root already saturated at the 4h harvest (Table 6). Fluctuations in the amount of ^{15}N-ammonium present in the root occurred as ^{14}N-ammonium, derived from the degredation of protein, diluted the ^{15}N-ammonium pools. The decrease in the ^{15}N-ammonium enrichment of the root between the 4h (79 A%E) and 8h (71A%E) harvest illustrates this.

At the 4h harvest the soluble ^{15}N content of the root was 1.5 times that of the shoot (Table 6 and Figure 6.1a). At the 8h harvest, however, the ^{15}N contents of the soluble and bound nitrogen fractions of the shoot exceeded those of the root (1.5 times and 1.2 times respectively). This suggests that the root is the main site of nitrogen assimilation when ammonium is supplied as the sole nitrogen source. Once the ammonium nitrogen has been assimilated into an organic form in the root, it is transported to the shoot in the xylem stream. Table 5 shows that the ^{15}N content of the amino acid fraction in the xylem stream increased 2.5 times between the 4h and 8h harvest. The ^{15}N-ammonium content of the xylem sap, however, was very small, the average value being 4.3ug ml^{-1} (Table 5). As already mentioned in Section 4.2.1., this reflects the apparent aversion of the plant to the accumulation of high levels of ammonium in its shoots as this could bring about the uncoupling of photosynthetic phosphorylation (Lewis et al., 1982). No nitrate was found in either the xylem sap (Table 5), or the plant extract as a whole.

The ^{15}N content of the soluble nitrogen pools in the shoot and root of ammonium-fed plants were considerably larger than those in nitrate-fed plants (Tables 6 and 4, Figures 6.1a and b). The average ^{15}N concentrations in the soluble nitrogen fraction of the shoot and root of ammonium-fed plants were 41.1 and 36.2 µg per plant respectively, compared with 11.4 and 3.6 µg per plant in nitrate-fed plants.

The bound ^{15}N levels in the shoot and root of ammonium-only fed plants (35.5 and 20.5 µg per plant) were also higher than those in nitrate-only fed plants (23.7 and 5.9 µg per plant), as can be seen from Tables 4 and 6, and Figures 6.1a and b.

With regard to the relative amounts of ^{15}N in the soluble and bound organic nitrogen pools within each feeding regime, it can be seen that, on the whole, in feeding regimes where the predominant nitrogen source was nitrate (the nitrate-only feed and the 3:1 nitrate:ammonium mixed feed) the ^{15}N concentrations in both the root and shoot, at the 4h and the 8h harvests, were highest in the bound nitrogen fraction (Tables 4 and 8, Figures 6.1b and d). An exception to this pattern occurred in the nitrate-only fed plants at the 4h harvest where the shoot ^{15}N content of the soluble nitrogen fraction (6.4 µg per plant) was slightly higher than that of the bound nitrogen fraction (5.3 µg per plant, Table 4 and Figure 6.1b). In the ammonium-only and the 1:1 nitrate:ammonium feeding regimes, however, the ^{15}N concentration in both the root and shoot, at the 4h and 8h harvests, were highest in the soluble organic nitrogen fractions (Tables 6 and 7, Figures 6.1a and c). The exception here was in the 1:1 mixed feed plants at the 8h harvest where the root ^{15}N content of the bound nitrogen fraction (24.5 µg per plant) exceeded that of the soluble organic nitrogen fraction (22.4 µg per plant, Table 7 and Figure 6.1c). This pattern of ^{15}N accumulation in the soluble and bound nitrogen fractions of plants from each of the nutrient regimes can be attributed to the difference in the relative assimilation rates of the ammonium and nitrate ions. Ammonium is rapidly assimilated into organic compounds (as discussed later in this section) and a build up of soluble nitrogen occurs to levels above those in the bound nitrogen fraction. The rate of incorporation of nitrogen into organic compounds is much slower in predominantly nitrate-fed plants, and as a result there is no substantial accumulation of amino compounds in the roots and shoots.

At the 4h harvest the total organic ^{15}N content of both the 1:1 mixed feed plants (92.7μg per plant) and the 3:1 nitrate : ammonium mixed feed plants (97.0 μg per plant), were higher than those of the ammonium-fed plants (80.0μg per plant) as can be seen from Figure 7. At the 8h harvest, however, the total organic ^{15}N content of the ammonium-fed plants, with a value of 198.1μg per plant, exceeded that of the 3:1 nitrate : ammonium mixed feed plants (156.5μg per plant) but the total organic ^{15}N content of the 1:1 mixed feed plants was the highest of all (206.7 μg per plant).

The highest rate of ^{15}N incorporation into organic nitrogen was exhibited by the 1:1 mixed feed plants (24.8 μg $^{15}N\ h^{-1}$ per plant or 24.7 μg $^{15}N\ h^{-1}\ g^{-1}$ fresh weight) followed by the ammonium-fed plants (22.4 μg $^{15}N\ h^{-1}$ per plant or 30.9 μg $^{15}N\ h^{-1}\ g^{-1}$ fresh weight) and the 3:1 nitrate:ammonium mixed feed plants (21.9 μg $^{15}N\ h^{-1}$ per plant or 14.3 μg $^{15}N\ h^{-1}\ g^{-1}$ fresh weight). The nitrate-only fed plants exhibited the lowest rate of incorporation of ^{15}N into organic nitrogen (5.6 μg $^{15}N\ h^{-1}$ per plant or 6.3 μg $^{15}N\ h^{-1}\ g^{-1}$ fresh weight).

From amino acid analysis, glutamine was found to be the most abundant form of organic nitrogen in the xylem sap of ammonium-fed plants, forming 52% of the total organic nitrogen transported in the xylem stream

(Table 14). Asparagine was the second most abundant amino compound.

TABLE 5

^{15}N content of the nitrate, amino acid and ammonium fractions of the xylem sap of 20-day old barley plants fed separately 2mM ^{15}N nitrate, 2mM ^{15}N ammonium, a 1mM ^{15}N nitrate - 1mM ^{15}N ammonium mixture or a 1.5mM ^{15}N nitrate - 0.5mM ^{15}N ammonium mixture for 4h and 8h. (Duplicate extracts were pooled to provide sufficient nitrogen for ^{15}N determinations).

		^{15}N CONTENT ($\mu g\ ml^{-1}$)			
XYLEM SAP FRACTION	HARVEST (h)	NITRATE FEED	1:1 MIXED FEED	AMMONIUM FEED	3:1 MIXED FEED
NITRATE	4	54.9	35.7	0	100.8
	8	100.1	44.5	0	125.1
AMINO ACID	4	20.5	93.5	81.8	45.2
	8	60.5	236.1	204.6	58.7
AMMONIUM	4	*	*	3.4	*
	8	*	*	5.1	*

* Insufficient present for ^{15}N determination

TABLE 14

Glutamine as a percentage of total amino compounds, and asparagine content, of xylem sap collected from barley plants fed on either 2mM nitrate, 2mM ammonium, a 1:1 mixture of nitrate and ammonium or a 3:1 nitrate and ammonium mixture of the two nitrogen sources. Results are expressed in $\mu mol\ ml^{-1}$.

		FEEDING SOLUTION			
FRACTION	HARVEST (h)	NITRATE FEED	1:1 MIXED FEED	AMMONIUM FEED	3:1 MIXED FEED
GLUTAMINE	4	6.9	35.5	22.1	18.5
	8	9.7	74.3	30.8	4.3
TOTAL ORGANIC N	4	13.9	45.1	44.7	26.0
	8	19.7	90.5	56.9	8.0
% GLUTAMINE	4	49.6	78.7	49.4	71.2
	8	49.2	82.1	54.1	53.8
ASPARAGINE	4	0.6	3.5	18.3	3.2
	8	1.8	7.2	21.3	1.7

TABLE 15

^{15}N enrichment (A%E) of the nitrate, amino acid and ammonium fractions of the xylem sap of 20-day old barley plants fed separately with either 2mM ^{15}N nitrate, 2mM ^{15}N ammonium, a 1mM ^{15}N nitrate - 1mM ^{15}N ammonium mixture or a 1.5mM ^{15}N nitrate - 0.5mM ^{15}N ammonium mixture for 4h and 8h.(Duplicate extracts were pooled to provide sufficient nitrogen for ^{15}N determinations).

		FEEDING SOLUTION			
XYLEM SAP FRACTION	HARVEST (h)	NITRATE FEED	1:1 MIXED FEED	AMMONIUM FEED	3:1 MIXED FEED
NITRATE	4	51.8	28.2	0	51.5
	8	59.6	32.5	0	53.3
AMINO ACID	4	21.0	46.1	26.0	25.2
	8	29.1	46.1	30.0	27.8
AMMONIUM	4	*	*	20.8	*
	8	*	*	32.2	*

* Insufficient present for ^{15}N determination

4.2.3 Nitrate and Ammonium-fed Plants

4.2.3.1 1:1 Mixed Feed

The patterns of nitrogen uptake and assimilation seen in nitrate and ammonium-fed plants were reflected in this treatment where both nitrogen sources were supplied. Table 7 and Figure 6.1c show that 8 hours after the beginning of the feeding experiment, the ^{15}N-nitrate content of the shoot was more than double that in the root, indicating that the shoot is the main destination of root-assimilated nitrate.

The ^{15}N-nitrate enrichment of all the three plant regions approximately doubled between the 4h and 8h harvest (Table 7). The ^{15}N-nitrate enrichment of the shoot increased from 11.9 to 22.0 A%E, that in the shoot base + seed region from 8.7 to 17.0 A%E, and that in the root from 14.1 to 32.6 A%E. As previously noted in the nitrate-only fed plants (Section 4.2.1.) this suggests that none of the nitrate pools were saturated at the 8h harvest, and that the uptake of the nitrate ion was slow when compared to the uptake of ammonium.

The suppression of nitrate ion absorption by ammonium ions has been reported in experiments with barley (Rao and Rains, 1978; Lewis _et al._, 1982) wheat (Minotti, Craig and Jackson, 1969; Minotti, Williams and Jackson, 1969a and b) and maize (Schrader _et al._, 1972). In this experiment the average ^{15}N enrichment of the nitrate pools, between the 4h and 8h harvest, in 1:1 mixed feed plants was 30.4 A%E (Table 15). This value was lower than that in nitrate-only fed plants (55.7 A%E). In addition the ^{15}N-nitrate content of the shoot and root in 1:1 mixed feed plants was lower than that in nitrate-only fed plants, especially at the 4h harvest (Tables 4 and 7, Figures 6.1b and c). In the 1:1 mixed feed, the ^{15}N-nitrate content of the shoot at 4h and 8h was 0.4 and 14.1 µg per plant respectively, and of the root at 4h and 8h, 1.2 and 6.5 µg per plant. In nitrate-only fed plants the ^{15}N-nitrate content of the shoot at 4h and 8h was 5.7 µg and 16.7µg per plant, while the root values, at 4h and 8h, were 4.0 and 11.1 µg per plant. It could be deduced from this data that the uptake of nitrate ions was being suppressed by the presence of ammonium. It must be remembered, however, that the ^{15}N-nitrate pools are not static. They are constantly changing as nitrate is absorbed, transported and used up in the formation of amino compounds. Nitrate was also supplied at a lower concentration in the 1:1 mixed feed plants (1mM) than in the nitrate-only fed plants (2mM), making less nitrate available for absorption.

The ^{15}N-ammonium enrichment of the roots of 1:1 mixed feed plants after 4h was 95.6% (Table 7), indicating that the ^{15}N-ammonium pools were already saturated by the end of this feeding period. This again reflects the rapid uptake of the ammonium ion in comparison to nitrate ion uptake. Despite the lower ammonium concentration of the feeding solution supplied to these plants (1mM) in comparison to that supplied to ammonium-only fed plants (2mM), the average ^{15}N-ammonium content of the root (75.3 µg per plant, Table 7 and Figure 6.1c) was 2.8 times that in the root of ammonium-only fed plants (26.8µg per plant, Table 6 and Figure 6.1a). The possible stimulation of ammonium uptake due to the presence of nitrate ions is discussed in Chapter 5, Section 5.1.

There was no trace of ammonium in the xylem sap of these 1:1 mixed feed plants (Table 5). The low level of free ammonium in the shoot (4.6µg per plant on average) must therefore originate from the reduction of nitrate and the turning over of amino compounds in this region of the plant.

The accumulation of ^{15}N-ammonium in the roots of these plants, the lack of ammonium in the xylem stream, and the high amino acid content of the xylem sap (an average value of 164.8 µg ^{15}N ml^{-1}, Table 5), indicate that the root is the major site of ammonium assimilation. Glutamine represented 80.5% of the total organic nitrogen being transported to the shoot in the xylem stream (Table 14).

By the 4h harvest the ^{15}N contents of the soluble and bound organic nitrogen fractions of the shoot had already exceeded those of the root (Figure 6.1c and Table 7). This reversal of the situation in ammonium-fed plants (Table 6, Figure 6.1a) was probably due to the contribution made by nitrate assimilation, which appears to take place predominantly in the shoot. The continued contribution by nitrate to the assimilated nitrogen of the shoot at the 8h harvest was illustrated by the 3-fold increase in the ^{15}N organic nitrogen content of the shoot (147 µg ^{15}N per plant), over that in the root (47 µg ^{15}N per plant, Table 7). The ^{15}N organic nitrogen content of the shoot in ammonium-fed plants (120 µg per plant) showed only a 2-fold increase over that in the root (71 µg per plant, Table 6, Figure 6.1a). As already mentioned in Section 4.2.2., these robust plants of the 1:1 mixed feeding regime exhibited a higher rate of incorporation of ^{15}N into organic nitrogen (24.8 µg ^{15}N h^{-1} per plant or 24.7 µg ^{15}N h^{-1} g^{-1} fresh weight) than plants from any of the other three feeding regimes (Figure 7).

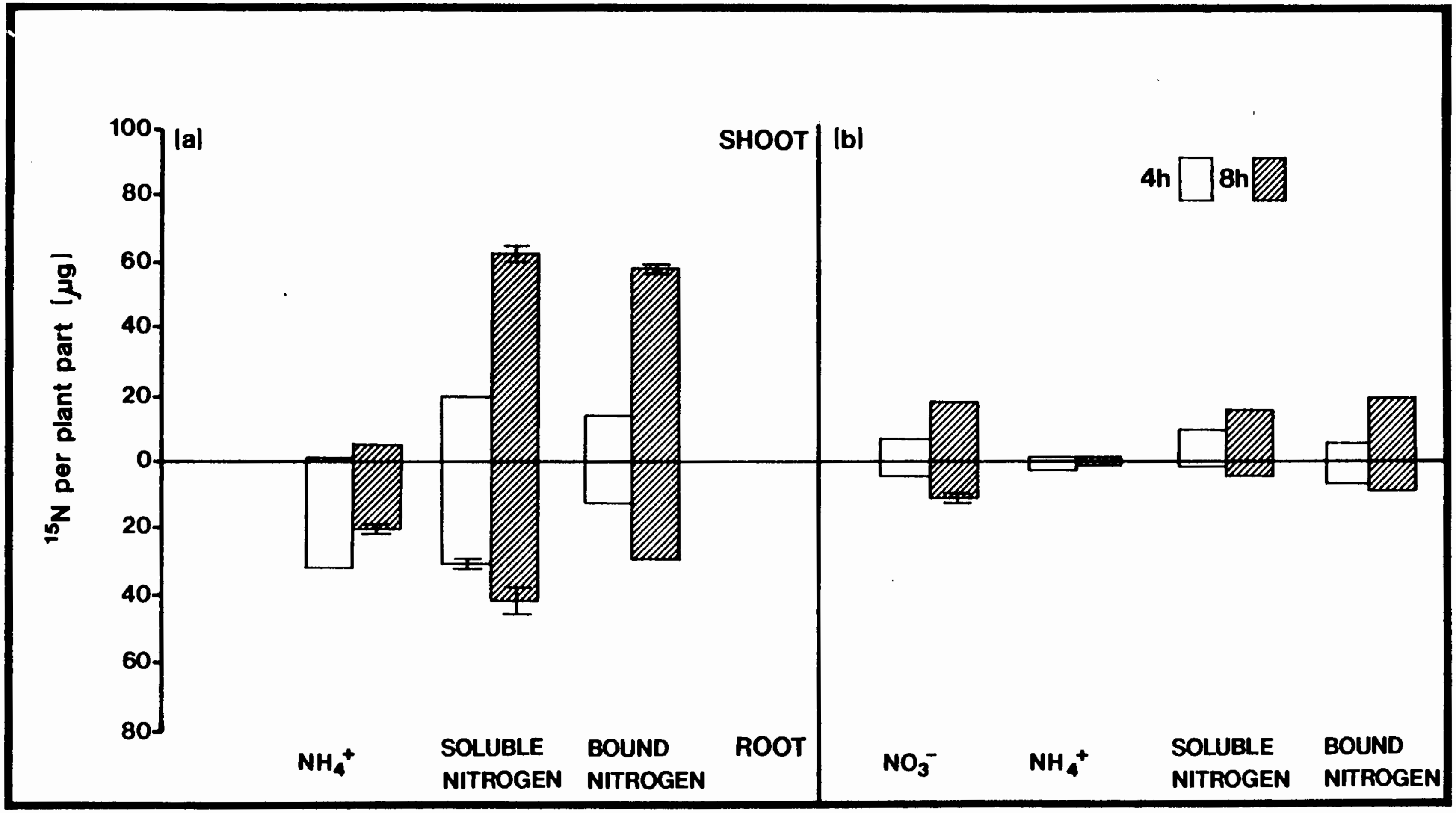

Figure 6.1

Accumulation of ^{15}N in nitrate and ammonium nitrogen, soluble nitrogen and bound nitrogen fractions of the root and shoot of individual 20-day-old barley plants fed separately with a) 2mM ammonium and b) 2mM nitrate and harvested 4h and 8h after the commencement of ^{15}N feeding. Bars represent mean and standard deviation of replicates. Where no bars appear, the difference between replicates is too small to be shown.

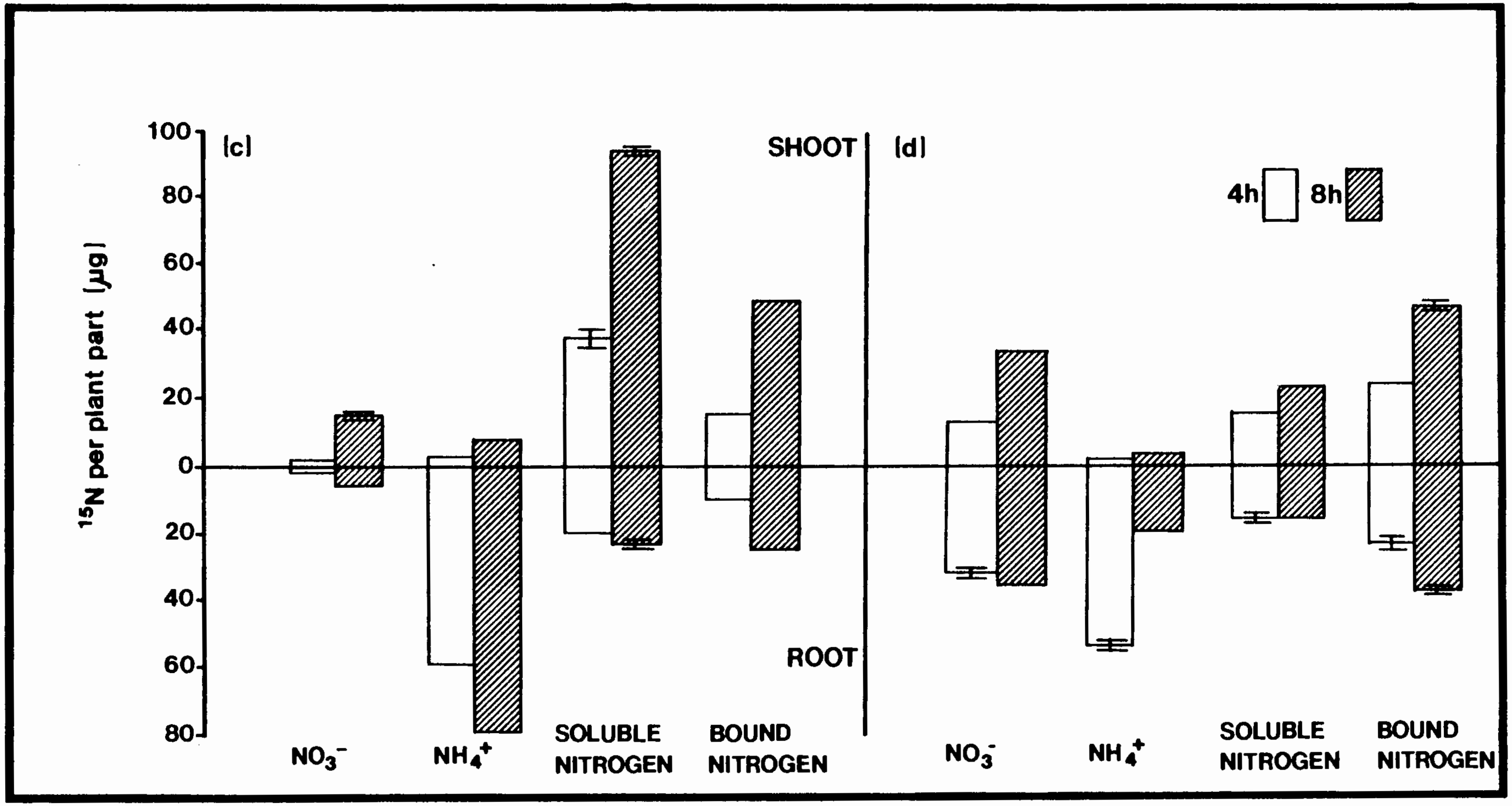

Figure 6.1 (cont).

Accumulation of ^{15}N in nitrate and ammonium nitrogen, soluble nitrogen and bound nitrogen fractions of the root and shoot of individual 20-day-old barley plants fed separately with c) 1:1 and d) a 3:1 nitrate:ammonium mixture, and harvested 4h and 8h after the commencement of ^{15}N feeding. Bars represent mean and standard deviation of replicates. Where no bars appear, the difference between replicates is too small to be shown.

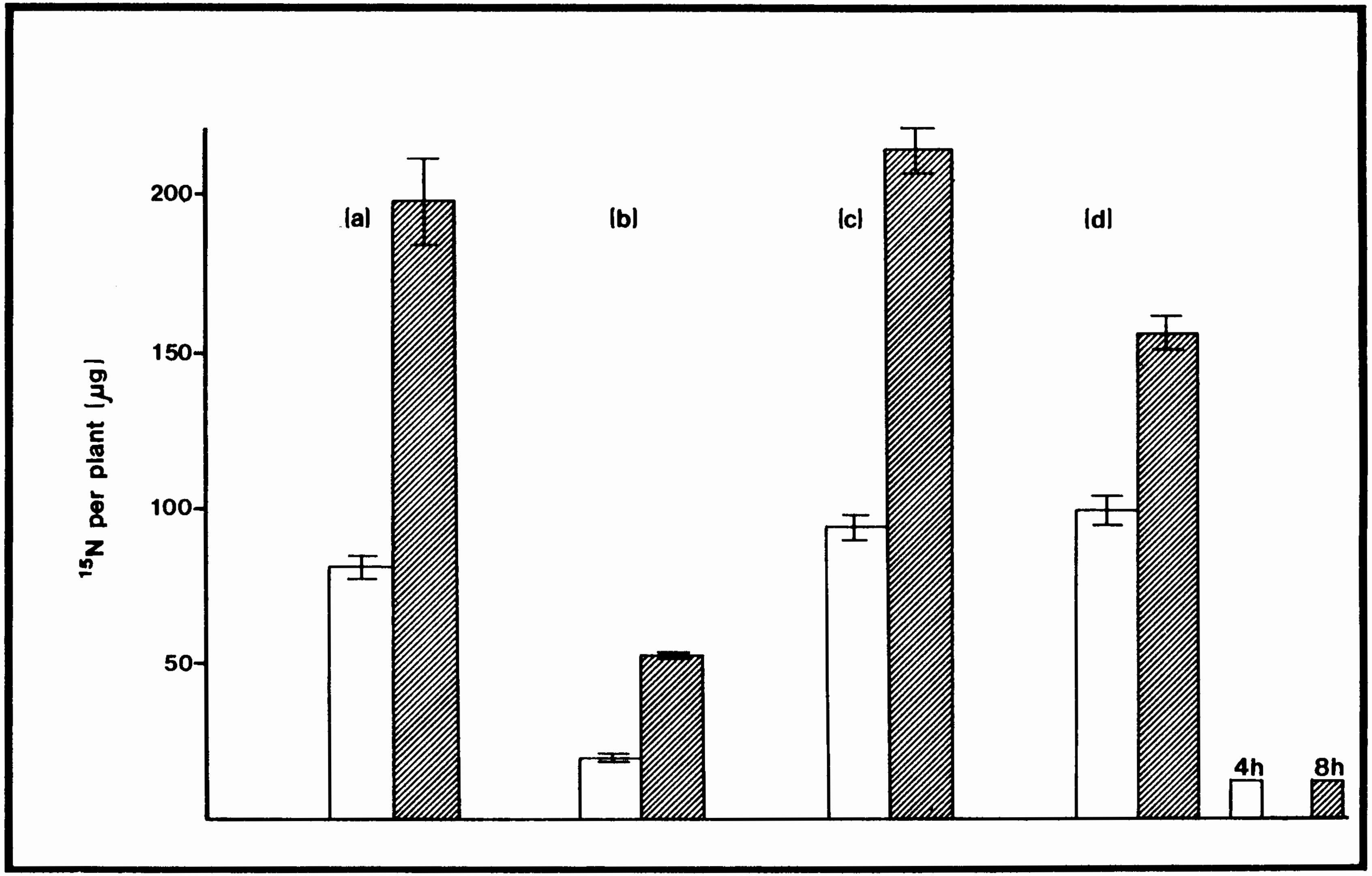

Figure 7

Total accumulation of ^{15}N in organic (i.e. assimilated) nitrogen of individual 20-day-old barley plants fed separately with either a) 2mM ammonium, b) 2mM nitrate, c) 1mM nitrate + 1mM ammonium mixture, or d) 1.5mM nitrate + 0.5mM ammonium mixture, and harvested 4h and 8h after the commencement of feeding. Bars represent mean and standard deviation of replicates. Where no bars appear, difference between replicates is too small to be shown.

4.2.3.2 3:1 Nitrate:Ammonium Mixed Feed

There was considerably more ^{15}N-nitrate in both the shoots and roots of 3:1 nitrate : ammonium mixed feed plants than in either the nitrate-fed (2.1 times more in the shoot and 4.4 times more in the root, Tables 4 & 8) or the 1:1 mixed feed plants (3.2 times more in the shoot and 8.6 times more in the root, Tables 7 & 8). The ^{15}N-nitrate pools of the root appear to be saturated at the 4h harvest with the 8h level (11 A%E) slightly lower than that at 4h (12A%E). This is in contrast to the pattern of ^{15}N enrichment in both the 1:1 mixed feed plants, where the ^{15}N-nitrate enrichment of the roots increased 2.3 times between the 4h and 8h harvest (Section 4.2.3.1) and the nitrate-fed plants, where it increased 2.8 times between the two harvests (Section 4.2.1).

In these 3:1 nitrate : ammonium mixed feed plants, however, the ^{15}N enrichment of the shoot nitrate increased from 5.3 to 12.7 A%E (a 2.4 fold increase, Table 8, Figure 6.1d) between the 4h and 8h harvest, demonstrating that at the 8h harvest ^{15}N-nitrate was still being absorbed by the roots and transported up to the shoot in the xylem stream. A similar increase in the ^{15}N enrichment of the shoot nitrate between the 4h and 8h harvest, was also observed in the 1:1 mixed feed plants, where the value increased 1.8 times, and the nitrate-only fed plants where the value increased 2.3 times (Tables 7 and 4 respectively).

The average ^{15}N-ammonium enrichment in the root was 72.3 A%E (Table 8). This value was similar to that in the ^{15}N-ammonium pools of the root in ammonium-only fed plants (75 A%E, Table 6). In these plants, in which ammonium formed the sole nitrogen source, the ion was rapidly assimilated in the root and the amino acids produced loaded onto the xylem stream (see Section 4.2.2). At the 8h harvest the ^{15}N content of the soluble organic nitrogen fraction of the shoot was 62.1 µg per plant (Table 6). Although not directly comparable, the 8h ^{15}N content of the soluble nitrogen fraction of the shoot in 3:1 nitrate : ammonium mixed feed plants was much lower (24.6 µg per plant, Table 8). These results suggest that nitrate inhibited ammonium assimilation in the 3:1 nitrate : ammonium mixed feed plants.

The ^{15}N content of the nitrate and amino acid fractions of the xylem sap in plants from each of the four nitrogen feeding regimes is shown in Table 5. The average ^{15}N nitrate : amino acid ratio in both the 3:1 nitrate : ammonium mixed feed plants and the nitrate-only fed plants was 2.2 : 1, showing that nitrate was the most common form of nitrogen being transported from root to shoot in the xylem stream.

In the 1:1 mixed feed plants the average ^{15}N nitrate : amino acid ratio in the xylem sap was 0.3 : 1 (derived from Table 5). Amino compounds originating from the assimilation of ammonium in the root, were the predominant nitrogenous compounds transported in the xylem stream in this feeding regime.

The ^{15}N content of the soluble organic nitrogen and bound nitrogen fractions in 3:1 nitrate:ammonium mixed feed plants showed an increase over that seen in the nitrate-only fed plants. At the 4h and 8h harvest respectively, the ^{15}N content of the soluble organic nitrogen fraction of the 3:1 mixed feed plants was 7.2 times and 3.3 times that in the root of nitrate-only fed plants, and 2.8 times and 1.5 times that in the shoot of nitrate-fed plants (Tables 8 and 4). The increased ^{15}N content in the bound nitrogen fraction was even more marked than in the soluble nitrogen fraction. At the 4h and 8h harvest respectively, the

^{15}N content of the bound fraction in 3:1 nitrate : ammonium mixed feed plants was 7.5 times and 4.3 times that in the roots of nitrate-fed plants, and 4.8 times and 2.7 times that in the shoot of nitrate-fed plants (Tables 8 and 4). The rate of incorporation of ^{15}N into organic nitrogen in 3:1 nitrate : ammonium mixed feed plants was 21.9 μg ^{15}N h^{-1} per plant or 14.3 μg ^{15}N h^{-1} g^{-1} fresh weight. The ^{15}N incorporation rate into organic nitrogen in nitrate-fed plants was much lower with a value of 5.6 μg ^{15}N h^{-1} per plant or 6.3 μg ^{15}N h^{-1} g^{-1} fresh weight.

As was found in xylem sap analysis of plants from the other three feeding regimes, glutamine was the most abundant amino compound being transported in the xylem stream of these 3:1 nitrate : ammonium mixed feed plants. This amide represented 62.3% of the total organic nitrogen in the xylem sap. Asparagine was the second most abundant amino compound (Table 14).

4.3 Nutrient Ammonium Assimilation by Barley Roots

4.3.1 ^{15}N Enrichment of Free Amino Compounds Without MSO Treatment

This investigation into ammonium assimilation by barley roots formed part of an ongoing experimental program in which I was not the only contributor. The GS and GDH enzyme assays in the presence and absence of MSO were carried out prior to my becoming involved in the program. It is essential, however, that the results of this work, as shown in Table 11, are included in this chapter in order that the project can be reviewed as a whole.

The assimilation of ^{15}N-ammonium into the most common free amino compounds of barley roots is shown in Table 9. The main routing of newly assimilated ^{15}N-ammonium, after both the 30 minute and 120 minute feeding period, was to glutamine with a secondary routing to glutamate. Aspartate, asparagine, serine and threonine were the other free amino compounds present in sufficient concentrations for ^{15}N analysis. These amino acids show far lower ^{15}N enrichments when compared with glutamine and glutamate.

4.3.2 ^{15}N Enrichment of Free Amino Compounds with MSO Treatment

As a result of this treatment there was a dramatic decrease in the incorporation of ^{15}N-ammonium into amino acids causing the ^{15}N-ammonium content of the root to increase 3.8 times (from 300 nmol g^{-1} fresh weight (Table 9) to 1136 nmol g^{-1} fresh weight (Table 10)). The amino acid pools were already greatly reduced at the end of the 30 minute feeding period with the ^{15}N glutamine content decreasing from 389 nmol g^{-1} fresh weight (Table 9) to a negligible value (Table 10).

TABLE 9

The ^{15}N enrichment of free amino compounds in the roots of 50 barley plants after feeding for 30 minutes and 120 minutes with a nutrient solution containing 2mM $^{15}NH_4Cl$.

	30 minute feeding time			120 minute feeding time		
AMINO COMPOUND	ROOT CONTENT (nmol g^{-1} fw)	^{15}N enrichment (A%E)	^{15}N CONTENT (nmol g^{-1} fw)	ROOT CONTENT (nmol g^{-1} fw)	^{15}N enrichment (A%E)	^{15}N CONTENT (nmol g^{-1} fw)
ASPARTATE	129	5.1 ± 0.2*	7 ± 0	213	10.2 ± 0.0	22 ± 0
ASPARAGINE	186	0.9 ± 0.1	3 ± 0	227	4.2 ± 0.2	19 ± 0
GLUTAMATE	428	16.4 ± 0.2	69 ± 2	471	22.4 ± 0.3	106 ± 1
GLUTAMINE	1408	13.8 ± 0.4	389 ± 11	1085	45.1 ± 0.2	979 ± 9
SERINE	80	2.7 ± 0.2	2 ± 0	127	3.9 ± 0.2	5 ± 0
THREONINE	35	0.0 ± 0.0	0 ± 0	51	2.9 ± 0.2	2 ± 0
AMMONIUM	1850	16.2 ± 0.3	300 ± 5	2300	20.2 ± 0.4	465 ± 9

* Mean and SD (three determinations)

TABLE 10

The ^{15}N enrichment of free amino compounds in the roots of 50 barley plants after feeding for 30 minutes and 120 minutes with a nutrient solution containing 2mM $^{15}NH_4Cl$ + 5mM MSO.

	30 minute feeding time			120 minute feeding time		
AMINO COMPOUND	ROOT CONTENT (nmol g^{-1} fw)	^{15}N enrichment (A%E)	^{15}N CONTENT (nmol g^{-1} fw)	ROOT CONTENT (nmol g^{-1} fw)	^{15}N enrichment (A%E)	^{15}N CONTENT (nmol g^{-1} fw)
ASPARTATE	149	0.2 ± 0.0*	0 ± 0	86	0.1 ± 0.0	0 ± 0
ASPARAGINE	215	0.3 ± 0.0	1 ± 1	112	0.1 ± 0.0	0 ± 0
GLUTAMATE	473	0.4 ± 0.2	2 ± 1	96	0.1 ± 0.0	0 ± 0
GLUTAMINE	59	0.2 ± 0.0	0 ± 0	22	0.2 ± 0.1	0 ± 0
SERINE	89	0.1 ± 0.0	0 ± 0	41	0.2 ± 0.1	0 ± 0
THREONINE	55	0.1 ± 0.0	0 ± 0	29	0.0 ± 0.0	0 ± 0
AMMONIUM	4304	26.4 ± 0.4	1136 ± 17	5977	29.9 ± 0.3	1787 ± 18

* Mean and SD (three determinations)

4.3.3 The Effect of MSO on Root Enzymes

4.3.3.1 Glutamine Synthetase and Glutamate Dehydrogenase

The activity of these enzymes, with and without MSO treatment, was investigated and the results are shown in Table 11. It is clear that GS activity is almost completely inhibited, while GDH activity is unaltered.

4.3.3.2 MSO plus ^{15}N-glutamine

To test the possibility of serious MSO interference with the activity of other enzymes associated with nitrogen assimilation, barley plants already treated with MSO were fed ^{15}N-amido glutamine plus MSO. The feeding and harvesting procedure is described in "Materials and Methods," Section 3.8.2 & 3.8.3. The plants were harvested after 120 and 240 minute feeding periods and the results are shown in Table 12.

In the amino acids present in sufficient quantities for ^{15}N analysis there was a marked increase in ^{15}N enrichment with time. This indicated that ^{15}N from the amido group of glutamine was being incorporated into these amino compounds despite the presence of MSO.

Table 11

Glutamine synthetase and glutamate dehydrogenase (NADH-dependent) activity of barley root in the presence and absence of methionine sulfoximine (MSO).

	Activity (umol h^{-1} g^{-1} FW)	
	- MSO	+ MSO
Glutamine synthetase	26.4 ± 1.5*	1.1 ± 0.1
Glutamate dehydrogenase	46.0 ± 1.6	49.0 ± 2.1

* Mean and standard deviation of activities of three extracts of the material fed ^{15}N for 30 minutes.

TABLE 12

The ^{15}N enrichment of free amino compounds in the roots of 50 barley plants after feeding for 120 minutes and 240 minutes with a nutrient solution containing 2mM ^{15}N-amido glutamine + 5mM MSO.

	120 minute feeding time			240 minute feeding time		
AMINO COMPOUND	ROOT CONTENT (nmol g^{-1} fw)	^{15}N enrichment (A%E)	^{15}N CONTENT (nmol g^{-1} fw)	ROOT CONTENT (nmol g^{-1} fw)	^{15}N enrichment (A%E)	^{15}N CONTENT (nmol g^{-1} fw)
ASPARTATE	137	*	*	149	5.2	8
ASPARAGINE	41	4.3	2	84	7.2	6
GLUTAMATE	86	3.2	3	224	5.1	11
GLUTAMINE	127	8.4	11	278	13.4	37
SERINE	35	0.7	0	61	1.5	1
ALANINE	54	2.7	2	85	3.9	3
GLYCINE	59	0.8	0	83	1.2	1

* Discharge tube failure

4.4 Six-week Growth Experiment: to Determine Fresh and Dry Weight and Total Nitrogen Content of Mature Barley Plants.

Barley plants were grown for six weeks and treated according to the techniques and environmental conditions outlined in Sections 3.2, 3.10.2 & 3.10.3. The nitrogen supplements used were either 2mM KNO_3, 2mM NH_4Cl or a 1mM KNO_3 plus 1mM NH_4Cl mixture.

From visual comparison of the plants grown on each nutrient regime (Plate 1) it was apparent that the 1:1 mixed feed plants were the largest, being on average 25% heavier than the nitrate fed-plants and 9% heavier than ammonium-fed plants. These figures were derived from Table 13. There were also differences in appearance between the plants from different feeding regimes although these were not as marked as those described for the 20-day-old seedlings (Section 4.1). The nitrate-fed plants were still, however, noticeably more fibrous than plants from the other treatments and the ammonium-fed plants more flaccid. The mixed nitrogen source plants were the most robust of the three treatments.

The average fresh and dry masses and the total nitrogen content of individual 6-week-old barley plants from each of the three feeding regimes are shown in Table 13. These results were derived from a mean of 60 plants per nitrogen source.

Although the mixed feed plants were larger and heavier than plants from the other two treatments the ammonium-fed plants had the highest total nitrogen content. With regard to the fresh masses of plants from the three nitrogen sources individual ammonium-fed plants were heavier than nitrate-fed plants.

On a total nitrogen per gram fresh weight basis the nitrate-only fed plants from the 6 week harvest show a much lower level of nitrogen assimilation than the 1:1 mixed feed plants, the figures being 3.70mg N g^{-1} fresh weight in the nitrate-fed plants and 6.20mg N g^{-1} fresh weight in the mixed feed plants. In the 20-day-old nitrate-fed plants, however, the total nitrogen per gram fresh weight figure was on a par with that for 1:1 mixed feed plants (Section 5.3). The reason for this must be related to the reduced nitrogen assimilation noted in 20-day-old nitrate-fed plants, and to the fact that the seed reserves in these mature 6-week-old plants would have been exhausted.

From the results presented in Tables 3 and 13 it can be seen that ammonium-fed plants have the highest total nitrogen per gram fresh weight values at both the 20-day harvest (4.02mg N g^{-1} fresh weight) and the 6-week harvest (7.93mg N g^{-1} fresh weight).

Average shoot and root fresh and dry weight ratios and whole plant fresh and dry weight ratios were derived from Table 13. The average shoot : root fresh weight of six-week-old ammonium-fed plants was 2.5:1, compared to 1.4:1 in nitrate-only fed plants and 1.9:1 in the 1:1 mixed feed plants. The increased shoot : root fresh weight value for plants from the ammonium-only nitrogen regime reflects the reduced growth of the roots of these plants, as described in Section 4.1.

The nitrate-fed plants had the lowest shoot : root dry weight value (2.4:1). This can be attributed to the dense and extensive root system observed in these plants. The ammonium-fed plants had the highest shoot : root dry weight value (5.3:1) with the value for the 1:1 mixed feed plants only slightly lower, at 4.6:1.

The average whole plant fresh to dry weight ratio of mature barley plants supplied with ammonium as their sole nitrogen source, was lower than that for plants from the other two feeding regimes. These values were 3.5 : 1, 7.1 : 1 and 4.0 : 1 for the ammonium-fed, nitrate-fed and 1:1 mixed feed plants respectively (derived from Table 13). This suggests that the ammonium-fed plants were in a lower state of turgor, and could partially explain the relative softness of ammonium-fed barley. In Chapter 5, Section 5.1, however, the characteristic growth forms of plants from the different nutrient regimes are discussed in terms of the way in which the two ions, ammonium and nitrate, are assimilated.

Plate 1

Six week old barley plants growing in nutrient solution containing either 2mM nitrate, 2mM ammonium or a 1mM nitrate : 1mM ammonium mixture.

TABLE 13

Total fresh and dry mass and total nitrogen content of whole plant, shoot + seed base and root regions of individual 6-week-old barley plants fed separately with 2mM nitrate, 2mM ammonium or 1mM nitrate : 1mM ammonium nutrient solutions.

	2mM NO_3^-			1:1 NO_3^- + NH_4^+			2mM NH_4^+		
	Total Fresh Mass (g)	Total Dry Mass (g)	Total N (mg)	Total Fresh Mass (g)	Total Dry Mass (g)	Total N (mg)	Total Fresh Mass (g)	Total Dry Mass (g)	Total N (mg)
WHOLE PLANT	1.21	0.17	4.48	1.51	0.38	9.36	1.38	0.39	10.94
SHOOT BASE + SEED	0.70	0.12	3.45	0.99	0.32	8.40	0.98	0.32	9.67
ROOT	0.51	0.05	1.02	0.51	0.07	0.96	0.39	0.06	1.27

Mean of 60 plants used per feeding treatment.

CHAPTER 5

GENERAL DISCUSSION AND CONCLUSIONS

5.1 ^{15}N Feeding Experiments

The extent to which nitrate and ammonium ions are absorbed, stored and assimilated by plants can vary considerably according to the plant species; indeed it has been reported that some plants can only tolerate one or other form of nitrogen (Krajina, Madoc-Jones and Mellor, 1973). For young barley plants it is apparent that a mixture of nitrate and ammonium is required for maximum growth and nitrogen assimilation. The contribution by nitrate to the increased nitrogen assimilation observed in the 1:1 mixed feeding regime, was to increase shoot nitrogen assimilation to levels above those seen in feeding regimes where only a single nitrogen source was supplied. The rapid uptake of the ammonium ion, and the faster rate of assimilation of ammonium into organic nitrogen in comparison to the rate of incorporation of the nitrate ion, form the basis of the contribution made by ammonium to the observed increase in nitrogen assimilation in mixed feed plants.

The reduced root production in ammonium-fed barley plants observed in this investigation has been noted previously (Warnke and Barber, 1973; Klem, 1966), and attributed to a pH decrease in the medium around the roots. Unless buffered, the absorption of ammonium brings about a decrease in the pH of the growth medium due to the concomitant release of H^+ ions. Maynard and Barker (1969), reported that the addition of calcium carbonate was effective in overcoming the poor development and brown colouration of ammonium-fed roots, but Cox and Reisenauer (1973), noted that roots were still short and thick in plants growing in a buffered ammonium solution at a pH of 5.4 ± 0.2 and that the leaves were very dark green with minute chlorotic spots. This is in agreement with the reduced growth and appearance of the leaves in barley plants even though the nutrient medium was maintained at a pH of 6.0 to 6.5 by the addition of calcium carbonate (Section 3.2).

The characteristic growth forms of the nitrate-only fed plants (with their erect, fibrous stems), and the ammonium-fed plants (with their soft and relatively flaccid stems), must be, to some extent, related to the way in which these ions are assimilated. From the enhanced growth observed when plants are supplied with both nitrate and ammonium as their nitrogen sources, it can be concluded that nitrate must stimulate growth in some way. It is possible that the presence of nitrate in the shoot stimulates photosynthesis.The subsequent incorporation of the concomitantly produced carbohydrate into cell wall components would account for the more robust nature of the stems of plants supplied with nitrate in their nutrient supply. In the case of ammonium-only fed plants, free ammonium is detoxified by being rapidly incorporated into amino compounds.
An abundant supply of photosynthetically-derived carbon skeletons is therefore required. The diversion of photosynthate from cell wall components into the production of carbon skeletons, and the channeling of the subsequently produced amino compounds into cytoplasmic constituents, would account for the more flaccid nature of ammonium-only fed plants. The robust, but supple growth form of plants supplied with both nitrate and ammonium indicates that a more balanced supply of constituents for cell growth were made available to the plant as a result of this feeding regime.

In many species, including barley, there is a close relationship between the main xylem component and the major soluble nitrogen storage compound (Pate, 1971). As well as acting as nitrogen-rich transport compounds, glutamine, asparagine and nitrate can act as storage forms of surplus nitrogen. In barley plants from nitrate-only and 3:1 nitrate:ammonium mixed feeding regimes, nitrate accumulated in the shoot and represented 66% and 81% respectively of the total ^{15}N transported in the xylem stream (Table 5). In contrast, amino acids and amides were the major components of the xylem sap in ammonium-only fed (97%) and 1:1 mixed feed (76%) plants (Table 5). The amides glutamine and asparagine were the predominant amino compounds. These organic compounds have a C:N ratio of less than 3:1. The roots rely on the shoots for carbon skeletons for nitrogen assimilation, and so the use of molecules with low C:N ratios for nitrogen transport allows the shoot to acquire nitrogen with the minimal export of carbon.

5.2 The Pathway of Ammonium Assimilation into Amino Compounds

The ^{15}N content and ^{15}N enrichment figures for free ammonium in the root of ammonium-fed plants (Table 9), show only a small increase between the 30 and 120 minute harvests, suggesting that by the 120 minute harvest this ammonium pool was saturated. A similar pattern of ^{15}N enrichment for ammonium has been observed by other workers (Bassham and Kirk, 1964; Lewis and Probyn, 1978; Probyn and Lewis, 1979; Kaiser and Lewis, 1980). This was regarded as evidence in favour of the existence of at least two pools of ammonium; a small, actively turning over assimilatory pool involved in the primary assimilation of newly absorbed ammonium, separated from a larger metabolic pool.

To preclude the possible participation of GDH in the assimilation of ammonium into amino acids, barley roots were supplied with ^{15}N-ammonium in the presence of MSO. The results, in Table 10, show that the incorporation of ^{15}N-ammonium into glutamate and amino acid assimilation has ceased and as a consequence the ^{15}N pool has increased greatly in size and ^{15}N enrichment. The free glutamine pool also diminished sharply in size indicating that virtually no incorporation of ^{15}N ammonium into this compound has taken place. It therefore appears that even when plants are supplied with ammonium as their sole nitrogen source, the GDH pathway does not play a substantial role in the assimilation of this compound in barley roots.

Various workers have previously reported on the specific inhibition of GS activity by MSO with no effect on NADH-dependent GDH (Brenchley, 1973; Lea and Norris, 1976; Miflin and Lea, 1976). The results of the GS and GDH assays shown in Table 11, and those of the ^{15}N enrichment of roots supplied with ^{15}N-glutamine and MSO (Table 12), confirm that MSO does not interfere with the activity of either GDH or other enzymes of nitrogen assimilation.

5.3 Six-week Growth Experiment

This growth experiment allowed a comparison to be made between 20-day-old barley seedlings and mature, 6-week-old plants with regard to their nitrogen assimilation and growth characteristics.

The plants from the mixed nitrogen source had the greatest total mass, and the ammonium plants the highest total nitrogen value when compared to plants from the other treatments in both the 20-day and the

6-week harvests. Individual ammonium-fed plants were, however, the smallest plants at 20 days old and the second largest at 6 weeks old. When the shoot and root fresh weights of ammonium-fed and 1:1 mixed feed plants are compared after 6 weeks growth (Table 13), it can be seen that the average shoot fresh weight of plants from both nitrogen regimes are equal. The average root fresh weight of ammonium-fed plants is, however, lower than that of the 1:1 mixed feed plants. The possible reasons for the reduced root growth observed in ammonium-only fed plants have already been discussed earlier in this chapter (Section 5.1).

It is interesting to note that in the 6-week growth experiment the highest root fresh weights were those from nitrate-only fed plants and 1:1 mixed feed plants (the value being 0.51g for both treatments), and the highest shoot fresh weights were those from the ammonium-only fed and 1:1 mixed feed plants (the values being 0.98g and 0.99g respectively). The robust mixed feed plants, therefore, possess a combination of the extensive root system characteristic of nitrate-only fed plants, and the relatively lush shoot system seen in the ammonium-only fed plants (Section 4.1).

In Section 4.2.1 it was reported that nitrate-fed plants showed a marked reduction in nitrogen assimilation compared to plants fed on ammonium or a mixed nitrogen source. In nitrate-fed plants from the 6-week growth experiment this observation is reflected in the total nitrogen per gram fresh weight values derived from Table 13. This value in nitrate-fed plants was 3.70mg N g^{-1} fresh weight and 6.20mg N g^{-1} fresh weight in mixed feed plants. In the 20-day harvest, however, the total nitrogen per gram fresh weight figure for nitrate-fed plants (derived from Table 3) was on a par with that for mixed feed plants, the figures being 2.70mg N g^{-1} fresh weight and 2.75mg N g^{-1} fresh weight respectively, (see Section 4.4).

5.4 Conclusions

1. In barley plants (Hordeum vulgare L.cv. Clipper) the fastest assimilation of inorganic nitrogen into an organic form was obtained in plants receiving a 1:1 mixed nitrate-ammonium nitrogen supply. The rates of ^{15}N incorporation into organic nitrogen exhibited by plants supplied with either nitrate or ammonium alone were 5.6 ± 0.2 μg ^{15}N h^{-1} per plant and 22.4 ± 1.0 μg ^{15}N h^{-1} per plant respectively. The rates for the mixed-nitrogen source plants were 24.8 ± 0.6 μg ^{15}N h^{-1} per plant (1:1 mixed feed plants) and 21.9 ± 1.0 μg ^{15}N h^{-1} per plant (3:1 mixed feed plants).

2. The increased ^{15}N assimilation noted in the 1:1 mixed feed plants, when compared to plants fed ammonium alone, can possibly be ascribed to the spacial separation of nitrate reduction and nitrate and ammonium assimilation within these plants. The process of nitrate assimilation in the shoot supplemented the contribution of ammonium assimilate from the root.

3. At the 4h harvest in ammonium-fed plants there was a higher accumulation of soluble organic nitrogen in the root than in the shoot indicating that the root is the major site of nitrogen assimilation when ammonium is supplied as the sole nitrogen source.

4. Once ammonium nitrogen has been assimilated into organic compounds in the root of ammonium-fed plants, it is transported to the shoot. Xylem sap analysis shows that 97% of the nitrogen exported from root to shoot in these plants is in the organic form, with very little ammonium present.

5. In this investigation into ammonium assimilation by barley roots the ^{15}N content and ^{15}N enrichment figures indicate the existence of at least two ammonium pools in the root. There is evidence for the presence of a small actively turning over assimilatory pool, concerned with the primary assimilation of newly absorbed ammonium, and a larger metabolic pool.

6. Xylem sap analysis, carried out on plants from the ^{15}N-nitrate feeding experiment, showed that 66% of the ^{15}N supply to the shoot was in the form of nitrate, the remainder being transported as organic nitrogen. This indicates that in 20-day-old barley plants the shoot is the main organ of nitrate assimilation.

7. A comparison between the ^{15}N enrichment of the xylem sap (52 A%E) and root tissue (4.6 A%E) of nitrate-fed plants at the 4h harvest demonstrates that newly absorbed nitrate largely bypasses the storage pools of the root and is loaded directly onto the xylem stream.

8. In this investigation the marked reduction in nitrogen assimilation observed when nitrate alone was supplied to plants as their nitrogen source, must be attributed mainly to the slower uptake of the nitrate ion when compared to ammonium.

9. From ^{15}N analysis of xylem sap, glutamine was found to be the most abundant form of organic nitrogen transported in the xylem stream, forming 49.7% of the total in nitrate-fed plants, 51.8% in ammonium-fed plants , 80.5% in the 1:1 mixed feed plants and 62.3% in the 3:1 mixed feed plants.

10. Plants from the 1:1 mixed feeding regime had significantly lower nitrate levels in the shoot, root and xylem sap than did plants supplied with 2mM nitrate in the feeding solution. This could either be attributed to the lower nitrate level of the 1mM nitrate + 1mM ammonium feeding solution, or to the suppression of nitrate uptake by ammonium ions.

11. The characteristic appearance of plants from the different nutrient regimes can be at least partially attributed to the degree to which carbon skeletons are incorporated into cellular components. In nitrate-fed plants the incorporation of an excess of photosynthetically-produced carbohydrate into cell wall components could give rise to the fibrous appearance of the stem. In ammonium-fed plants the contrasting softness of the stem may be due to the emphasis on the incorporation of carbon skeletons into nitrogen compounds. The mixed-feed plants display a combination of the characteristics seen in the single nitrogen source plants.

12. At the 20-day harvest, plants supplied with a mixed nitrogen source were larger than those fed on either nitrate or ammonium alone. The 3:1 mixed feed plants had the greatest total mass, being 53% heavier on average than those from the 1:1 mixed feed. The smallest plants, with the lowest total mass, were those supplied with ammonium only in the nutrient supply.

At the end of the 6-week growth experiment, in which only three nitrogen regimes were provided (2mM ammonium, 2mM nitrate and a 1mM nitrate + 1mM ammonium mixture) the mixed feed plants were still the largest and had the greatest total mass. The smallest plants, however, and those with the lowest total mass, were the nitrate-only fed plants.

13. In barley roots ammonium was shown to be assimilated entirely via the glutamine synthetase/glutamate synthase (GS/GOGAT) pathway, with glutamate dehydrogenase (GDH) playing an insignificant role in the process.

14. Root enzyme assays and the ^{15}N enrichment of roots supplied with ^{15}N-glutamine and MSO (Tables 11 and 12 respectively), demonstrate that GS is completely inhibited by treatment with methionine sulfoximine, while the activity of GDH, GOGAT and various other enzymes of nitrogen assimilation remains unaffected.

15. The results of this investigation show that for optimal growth and nitrogen assimilation of hydroponically grown "Clipper" barley, a mixed nitrate-ammonium nitrogen regime is preferred. Field grown plants could show different responses to the nitrogen sources supplied in this project, however, since certain soils, notably those containing large quantities of micaceous clay minerals (vermiculite, illite and montmorillonite) fix ammonium nitrogen, rendering it unavailable to plants. Much work has been done with the objective of keeping added ammonium fertilisers in the ammonium form and inhibiting nitrification, or nitrate formation. The use of nitrification inhibitors could, in the future, lead to an improvement in the nutritional quality and quantity of important crop plants.

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