AMMONIUM UPTAKE BY RICE ROOTS

by

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ABSTRACT

$^{13}NH_4^+$ uptake was studied using 3-week-old rice plants (*Oryza sativa* L. cv. M202), grown hydroponically in modified Johnson's nutrient solution containing 2, 100 or 1000 μM NH_4^+ (referred to hereafter as G2, G100 or G1000 plants, respectively). At steady-state, the influx and efflux of $^{13}NH_4^+$ was increased as NH_4^+ provision during growth was increased. The half-life of cytoplasmic $^{13}NH_4^+$ exchange was calculated to be 8 min while the half-life for cell wall exchange was 1 min. Cytoplasmic [NH_4^+] of G2, G100 and G1000 roots was estimated to be 3.72, 20.55, and 38.08 mM respectively. However about 72% to 92% of total root NH_4^+ was located in the vacuole. During a 30 minute period G100 plants metabolized 19% of the newly absorbed $^{13}NH_4^+$ and the remainder was partitioned among the cytoplasm (41%), vacuole (20%) and efflux (20%). Of the metabolized ^{13}N, roughly one half was translocated to the shoots.

In short-term, perturbation experiments, below 1 mM external concentration ($[NH_4^+]_o$), $^{13}NH_4^+$ influx of G2, G100 and G1000 roots was saturable and operated by means of a high affinity transport system (HATS). The V_{max} values for this transport system were negatively correlated and K_m values were positively correlated with NH_4^+ provision during growth and root [NH_4^+]. Between 1 and 40 mM $[NH_4^+]_o$, $^{13}NH_4^+$ influx showed a linear response to external concentration due to a low affinity transport system (LATS). The $^{13}NH_4^+$ influxes by the HATS, and to a lesser extent the LATS, are energy-dependent processes. Selected metabolic inhibitors reduced influx of the HATS by 50 to 80%, but of the LATS by only 31 to 51%. Estimated Q_{10} values for HATS were greater than

2.4 at root temperatures from 5 to 10°C and constant at ~1.5 between 5 to 30°C for the LATS. Influx of $^{13}NH_4^+$ by the HATS was insensitive to external pH in the range from 4.5 to 9.0, but influx by the LATS declined significantly beyond pH 6.0.

The transmembrane electrical potential differences ($\Delta\Psi$) of epidermal and cortical cells of intact roots were in the range from -120 to -140 millivolts (mV) in the absence of NH_4^+ in bathing solution and were -116 mV and -89 mV for G2 and G100 plants in 2 and 100 μM NH_4^+ solutions, respectively. Introducing NH_4^+ to the bathing medium caused a rapid depolarization which exhibited a biphasic response to external [NH_4^+]. Plots of membrane depolarization versus $^{13}NH_4^+$ influx were also biphasic, indicating distinct coupling processes for the two transport systems, with a break-point between the two concentration ranges around 1 mM NH_4^+. Depolarization of $\Delta\Psi$ due to NH_4^+ uptake was eliminated by a protonophore (carboxylcyanide-*m*-chlorophenylhydrazone), inhibitors of ATP synthesis (sodium cyanide plus salicylhydroxamic acid), or an ATPase inhibitor (diethylstilbestrol).

$^{13}NH_4^+$ influx was regulated by internal ammonium and its primary metabolites, amides and amino acids. When internal amide or amino acids concentrations were increased, the influx of $^{13}NH_4^+$ was reduced. However, treating rice roots with L-Methionine DL-Sulfoximine (MSX) reduced the levels of ammonium assimilates but did not increase $^{13}NH_4^+$ influx probably because internal [NH_4^+] was increased. Short-term nitrogen depletion stimulated $^{13}NH_4^+$ influx, but long-term N depletion caused NH_4^+ influx to be reduced probably due to N limitation of carrier synthesis. A cascade regulation system is proposed to explain the multi-level regulation of NH_4^+ influx.

The interaction between ammonium and potassium showed that when N is adequate, K promoted NH_4^+ uptake and utilization. Likewise, proper N nutrition promoted K^+ uptake but the presence of NH_4^+ in uptake solution strongly inhibited the K^+ ($^{86}Rb^+$) uptake at the transport step. The results indicated that NH_4^+ and K^+ may share the same channel but are regulated by different feedback signals.

TABLE OF CONTENTS

Abbreviations

AA amino acids
AFS Appearent free space
AOA amino-oxyacetate
Arg Arginine
Asn Asparigine
Asp Aspatarte
Azaserine O-diazoacetyl-L-serine;
CCCP carboxylcyanide-*m*-chlorophenyl-hydrazone;
CN^- (sodium) cyanide;
DES diethylstilbestrol;
DMRT Duncan's multiple range test.
DNP 2,4-dinitrophenol;
DON 6-diazo-5-oxo-L-norleucine;
$\Delta\Psi$ transmembrane electrical potential difference;
Φ_{ass} rate of assimilation of $^{13}NH_4^+$ in roots;
ϕ_{cv} flux across the tonoplast into vacuole;
Φ_{cx} translocation of ^{13}N labeled metabolites to xylem (shoots);
ϕ_{oc}, ϕ_{co}, and ϕ_{net} inward, outward and net fluxes (μmol g^{-1}FW h^{-1}) across the plasmalemma, respectively;
G2, G100 and G1000 plants rice seedlings grown in MJNS containing 2, 100 or 1000 μM NH_4^+, respectively;
G2M, G100M and G1000M MJNS containing 2, 100 or 1000 μM NH_4^+, respectively, as growth media;
GDH glutamate dehydrogenase *(GDH; EC 1.4.1.2)*
Gln Glutamine
Glu Glutamate
GOGAT glutamate synthase;
GS glutamine synthetase;
HATS or LATS high affinity or low affinity NH_4^+ transport systems, respectively;
K_m the external ion concentration giving half of the maximum rate (μM);
LSD Least significant difference;
MA methylamine
MJNS modified Johnson's nutrient solution;

MSX	L-Methionine DL-Sulfoximine
NiR	nitrite reductase
NR	nitrate reductase
*p*CMBS	*p*-chloromercuribenzene-sulfonate;
Q_i, Q_c, Q_v	ammonium contents (μmol g^{-1}FW) of root, cytoplasm and vacuole, respectively;
SHAM	salicylhydroxamic acid;
S_o and S_c	radioisotopic specific activities of external media and cytoplasmic compartments, respectively;
V_{max}	the calculated maximum rate of ion influx (μmol g^{-1}FW h^{-1});
$[NH_4^+]_c$	cytoplasmic ammonium concentration (μM or mM);
$[NH_4^+]_i$	root (internal) ammonium concentration (μM or mM);
$[NH_4^+]_o$	external ammonium concentration (μM or mM);
$[NH_4^+]_v$	vacuolar ammonium concentrations (μM or mM);

List of Tables

List of Figures

To my wife, Xiao Ge

for her love, understanding and sacrifice

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Chapter 1. RESEARCH BACKGROUND

1.1. General Introduction

1.1.1. Rice

Rice (*Oryza sativa* Linneaus) is a semi-aquatic, annual grass plant in the family *Poaceae* (formerly *Graminae*). Rice is grown in over 100 countries on every continent except Antarctica, extending from 53°N to 35°S latitude, from sea level to 3000 m altitude (Lu and Chang, 1980; Mikkelsen and De Datta, 1991). Rice grows either as an upland (dry) or lowland (wet) crop in the tropic, subtropics, temperate, and subtemperate zones and on plains, hilly regions, and plateaus. About 53% of total land area under rice cultivation is irrigated, producing 73% of the world's rice (De Datta, 1988). More than 90% of the world's rice is produced in Asia (IRRI, 1988). Rice is the staple food and the energy source for about 40% of the world's population (De Datta, 1981, 1986b); it supplies the energy source for more than half of the world's population and provides 75% of the caloric intake of Asia's over two billion people (Buresh and De Datta, 1991).

1.1.2. Essentiality of nitrogen

Nitrogen is required for the synthesis of amino acids, proteins, nucleic acids and many secondary plant products such as alkaloids. It is involved in the whole life cycle of plants; in enzymes for biochemical

processes, in chlorophyll for photosynthesis, and in nucleoproteins for the control of hereditary and developmental processes. Since N is present in so many essential compounds, it is not surprising that growth without sufficient N is slow. Nitrogen is the single most important chemical element limiting crop yield.

1.1.3. Necessity of N fertilization

Proper application of N increases both yield and protein content of rice (Patrick et al., 1974; Gomez and De Datta, 1975; Allen and Terman, 1978). The intensification of rice production has involved a tremendous increase in the use of nitrogen fertilizers and the selection of high yielding varieties that are highly responsive to nitrogen. However, research on the effects of nitrogen fertilizers on rice production has focused mainly on the agronomic context, in terms of grain yield, carbohydrate metabolism, growth patterns or morphological characteristics. Information concerning physiological and biochemical aspects of nitrogen uptake by rice as well as other higher plants, is limited, which is unfortunate since these details may prove to be important for the production of new varieties with improved nitrogen utilization.

1.1.4. Bio-availability of nitrogen

Ammonium is the predominant and most readily bio-available nitrogen form in paddy soil; it is the preferred nitrogen species taken up by rice plants (Sethi, 1940; Sasakawa and Yamomoto, 1978; Goyal and Huffaker, 1984). Besides NH_4^+, rice roots also absorb NO_3^- (Malavolta,

1954) and organic nitrogen such as urea, Gln and Arg (Arima and Kumazawa, 1977; Mori et al., 1979; Mori and Nishizawa, 1979; Harper, 1984).

1.2. AMMONIUM UPTAKE

1.2.1. Importance of transport research

Information on the ammonium transport system(s) of root cells of rice, and their regulations, is meagre. Moreover, the relationships among uptake, assimilation and other metabolic processes are not as well understood as is the case for other plant nutrients. To understand the ammonium transport system(s), generally, it is necessary to characterize their kinetics, energetic and genetic properties. In order to achieve this, fluxes should be measured in response to variation of concentration, temperature and pH, and the effects of metabolic inhibitors should be determined. Where transport mutants are available, the genetic basis of the transport system(s) can be evaluated (Kleiner, 1981, 1985; Glass, 1988). This information satisfies more than the researcher's curiosity; it provides a better understanding of ammonium uptake for the development of better fertilization practice and improved variety selection.

1.2.2. Transport of NH_4^+ by lower plants

Ammonium uptake has been well studied in bacteria, fungi and algae (Kleiner, 1975, 1981, 1985; Roon et al., 1977; Pelley and Bannister, 1979; Smith and Walker, 1978; Boussiba et al., 1984). In brief, ammonium can be accumulated against its concentration and electrochemical potential gradients, resulting in significant ammonium concentration within plant cells (Smith and Walker, 1978; Pelley and Bannister, 1979; Kleiner, 1981; Boussiba et al., 1984). NH_4^+ uptake is concentration dependent and its isotherm in the low range of external concentration conformed to Michaelis-Menten kinetics (Hackette et al., 1970; Dubois and Grenson, 1979; Felle, 1980; Fuggi et al., 1981; Smith, 1982; Box, 1987). NH_4^+ transport across the plasma membrane has been claimed to occur via an electrogenic uniporter which depolarizes membrane electrical potential differences (Barr et al., 1974; Haines and Wheeler, 1977; Slayman, 1977; Raven and Smith, 1976; Smith et al., 1978; Smith and Walker, 1978; Walker et al., 1979a, 1979b; Laane, 1980; Raven, 1980; Smith, 1980; Kleiner and Fitzke,1981; Bertl et al., 1984; Ullrich et al., 1984). The Q_{10} value for NH_4^+ uptake has been reported to be ~ 2.0 (Hackette et al., 1970) and ATP may be involved in the transport step, hence the uptake system is inhibited by anaerobiosis or several metabolic inhibitors (Stevenson and Silver, 1977; Cook and Anthony, 1978a; Felle, 1980). The responses of NH_4^+ uptake to pH changes is complex (Hackette et al., 1970; Roon et al., 1977; Kleiner, 1981). The optimum pH was 6~7 for bacteria and fungi. The existence of specific proteinaceous carriers for NH_4^+ uptake is supported by biochemical, kinetic and physiological evidences. Moreover, NH_4^+ transport mutants have been isolated and some transport genes have been identified and cloned (Arst and Page, 1973; Castorph and Kleiner, 1984; Holtel and Kleiner, 1985; Franco et al., 1987; Reglinski et al., 1989).

1.2.3. Transport of NH_4^+ by higher plants

There is a limited literature available regarding NH_4^+ transport in higher plants (Highinbotham et al., 1964), although a number of kinetic studies were reported for NO_3^- uptake (Deane-Drummond and Glass, 1982a, 1983a; Siddiqi et al., 1990; Hole et al., 1990; Wieneke, 1992). Generally the NH_4^+ transport systems in higher plants are very similar to those in lower plants. Ammonium transport is localized perhaps at the plasma membrane and possible other membranes (Kleiner, 1981; Churchill and Sze, 1983). Evidence from kinetic studies of ammonium uptake by plant roots indicates that NH_4^+ transport is a carrier-mediated process (Nissen, 1973; Joseph et al., 1975). There are several lines of evidence that support the existence of the proteinaceous carriers to be discussed in the following sections.

1.2.3.1. Carrier-mediated transport

Evidence indicating that ammonium transport is a carrier-mediated process (Nissen, 1973; Joseph et al., 1975) comes from determining kinetic parameters for NH_4^+ accumulation in cells (Kleiner, 1985). The uptake of NH_4^+ by barley, rice, ryegrass, tomato, and wheat is concentration dependent and follows Michaelis-Menten kinetics, (Tromp, 1962; Lycklama, 1963; Fried et al., 1965; Cox and Reisensuer, 1973; Rao and Rains, 1976; Bloom and Chapin, 1981; Youngdahl et al., 1982; McNaughton and Presland, 1983; Bloom, 1985; Deane-Drummond and Thayer, 1986; Smart and Bloom, 1988). Presland and McNaughton (1986) examined the rates of NH_4^+ uptake as a function of external $[NH_4^+]$ in corn, and reported a saturable system below 1 mM $[NH_4^+]$. In a continuously flowing nutrient solution system, NH_4^+ uptake rates of intact rice plants were fitted to a Michaelis-Menten model (Fried et al., 1965; Youngdahl et al., 1982).

1.2.3.2. Concentration dependent kinetics

A biphasic pattern of NH_4^+ uptake, with both saturable and linear phases, was first reported in *Lemna*, by Ullrich et al., (1984). For crops, like corn, and rice, NH_4^+ uptake kinetics (below 1 mM $[NH_4^+]_o$) commonly conform to Michaelis-Menten patterns with K_m values ranging from 0.014 to 0.167 mM (Fried et al., 1965; Youngdahl et al., 1982; Presland and McNaughton, 1986; Glass, 1988). K_m values of 0.075 and 0.103 mM and a V_{max} of 0.061 and 0.017 mmol kg^{-1} s^{-1} were obtained for 4-week-old and 9-week-old rice plants, respectively, (Youngdahl et al., 1982). The second system, above 1 mM $[NH_4^+]$, failed to correspond to Michaelis-Menten kinetics (Ullrich et al., 1984; Presland and McNaughton, 1986). Generally, uptake studies at high external concentration have been achieved only with some difficulty, because depletion of the external solution is so small.

1.2.3.3. Depolarization of membrane potential

The inward movement of ammonium occurs as the cation NH_4^+ (Walker et al., 1979a, 1979b; MacFarlane and Smith, 1982; Kleiner, 1985; Deane-Drummond, 1986). Only one report measuring $\Delta\Psi$ in rice roots has appeared in the literature: Usmanov (1979) reported $\Delta\Psi$ to be -160 mV. As early as 1964, Higinbotham et al., noted the marked depolarizing effect of $[NH_4^+]_o$ on coleoptile cells $\Delta\Psi$ in oats. Ullrich et al., (1984) found that, in *Lemna*, depolarization of $\Delta\Psi$ by NH_4^+ below 0.2 mM $[NH_4^+]_o$ was concentration-dependent and both NH_4^+ uptake and $\Delta\Psi$ depolarization responded in a saturable fashion with half saturation values of 17 μM for both processes. From 0.2 to 1 mM, net uptake of NH_4^+ responded linearly to $[NH_4^+]_o$, with no further $\Delta\Psi$ depolarization. Since NH_4^+ is the main species taken by plant roots, it must be taken up via active transport and/or

facilitated diffusion. Both processes are coupled to an energy source, either directly (the former) or indirectly (the latter).

1.2.3.4. Energy dependence

Metabolic energy is important to NH_4^+ uptake. Macklon et al., (1990) has shown that NH_4^+ absorption by excised root segments of *Allium cepa* L. was an active process. The uptake of ammonium at high temperature (25-30°C) is closely associated with metabolism (Sasakawa and Yamamoto, 1978), and the uptake process was also decreased when carbohydrate levels were reduced (see Section 1.3.1.1.) or when temperatures were lowered (see Section 1.3.2.1.).

1.3. MAJOR FACTORS AFFECTING AMMONIUM UPTAKE

Besides the mechanism and kinetics of ammonium uptake, research on ammonium uptake has also included other related issues such as the effect of energy status, nitrogen cycling within the plant, the effects of root pH and temperature. It must be emphasized that when environmental factors are concerned, one must be aware of the root's capacity to adapt ion uptake in response to changed conditions, especially in long-term experiments.

1.3.1. Effects of photosynthesis

1.3.1.1. Dependence on soluble carbohydrates

Of major importance in the uptake of ammonium is the energy status of the plant. The energy status of rice plants had a substantial influence on

the uptake of NH_4^+ and on its conversion into high molecular weight N compounds (Mengel and Viro, 1978). The high demand for carbohydrate is in order to achieve active transport of NH_4^+ at low external concentration, and to supply carbon skeletons for the rapid assimilation of NH_4^+ as it is absorbed by roots (Givan, 1979; Fentem et al., 1983a, 1983b). When the availability of carbohydrate is low, the assimilation of NH_4^+ is also low (Breteler and Nissen, 1982), and consequently a high efflux rate of NH_4^+ may result. A general relationship exists between the proportion of total nitrogen absorbed as NH_4^+ from mixed N sources such as NH_4NO_3 and the availability of soluble carbohydrates in roots. (Raper et al., 1992). The concentration of soluble carbohydrates in the leaves of NH_4^+-fed plants was greater than that of NO_3^--fed plants, but was lower in roots of NH_4^+-fed plants, regardless of pH (Chaillou et al., 1991).

The study of NH_4^+ uptake isotherms in *Chlorella* revealed that preincubation with glucose drastically increased V_{max} (5-fold), with no change of K_m (Schlee and Komor, 1986). It was reported that glucose induced a glucose transport system and two specific amino acid transport systems (Cho et al., 1981). Glucose also induced the transport systems for ammonium, nitrate and urea (Schlee et al., 1985). Removal of the endosperm of rice seedling suppressed NH_4^+ uptake markedly (Sasakawa and Yamamoto, 1978), while the addition of 30 mM sucrose restored uptake. In higher plants, provision of carbon skeletons in the form of α-ketoglutarate increased uptake and association of NH_4^+ in *Lemna* (Monselise and Kost, 1993).

1.3.1.2. Periodic variations of light and growth

There is a great variation in NH_4^+ assimilation rates between day and night during the tillering stage of rice plants (Ito, 1987). This is probably

related to the diurnal changes in carbohydrate flux from shoot to root resulting from changes in relative source-sink activity of shoots (Rufty et al., 1989; Lim et al., 1990). This periodic variation of carbohydrate supply is also influenced by morphological variations of plant growth (Henry and Raper, 1989a; Vessey et al., 1990b). The net rate of NH_4^+ uptake oscillated between a maximum and a minimum with a periodicity co-ordinate with intervals of leaf emergence (Tolley and Raper, 1985; Tolley-Henry et al., 1988; Henry and Raper, 1989a; Rideout et al., 1994). Changes of both influx and efflux were responsible for the observed differences of net NH_4^+ uptake (Henry and Raper, 1989).

1.3.1.3. Ambient environmental factors

The ability of the plant root to absorb nitrogen was affected by previous growth conditions of the examined plants (Mori et al., 1979), since environmental factors will influence the carbohydrate status. Susceptibility of plants to NH_4^+ toxicity is also related to plant carbohydrate status (Nightingale, 1937; Prianishnikov, 1941; Givan, 1979). The soluble carbohydrate concentration in roots increased with increasing root temperature (Clarkson et al., 1975; Macduff et al., 1987a) and with nitrogen deprivation (Rufty et al., 1988; Henry and Raper, 1991), and decreasing rhizospheric pH (Chaillou et al., 1991). High ambient CO_2 concentration increased total plant N and total nitrate-N content and leaf area but not leaf number of soybeans.

1.3.2. Effects of root temperature

1.3.2.1. Short-term perturbation

Ammonium transport across the plasma membrane is sensitive to temperature. Although ion accumulation at steady-state may be independent of external concentration or temperature, both of these factors influence short-term fluxes (Cram, 1973; Smith, 1973, Glass, 1983). In a 5-hour root temperature perturbation study, it was found that the uptake and assimilation of ammonium were profoundly affected in both *Indica* and *Japonica* rice plants (Ta and Ohira, 1981). This might be explained by the dependence of the NH_4^+ uptake system on the rate of metabolism (Raven and Smith, 1976), or effects of low temperature on enzymes of NH_4^+ assimilation (Shen, 1972). The effect of temperature on ion uptake may also be due to physical changes in different parts of the cell membrane (e.g. membrane fluidity) instead of on the transport process (Clarkson and Warner, 1979).

1.3.2.2. Q_{10} value for NH_4^+ uptake

Q_{10} values can be used to indicate the temperature dependence of ion transport. When temperature is lowered or increased by 10°C, the ratio of the two transport rates can be calculated by equation:

$$\text{Ln } Q_{10} = [(t_2 - t_1)/10\,]\ \text{Ln}\ (V_2 / V_1) \qquad [1]$$

where t_1 and t_2 are the temperature before and after the change, and V_1 and V_2 are the transport rates at respective temperatures. When Q_{10} is close to 1, the transport rates are the same at the different temperatures, and ion transport is insensitive to temperature. A Q_{10} value greater than 2 is often considered as indicating the metabolic dependence of a

physiological process such as ion transport. In a seven hours perturbation of root temperature, Sasakawa and Yamamoto (1978) found that the uptake of ammonium by 9-days old rice seedlings was closely associated with metabolism. The Q_{10} values between 9 ~ 24°C were > 2.5 for $^{15}NH_4^+$ absorption by rice roots estimated from Ta and Ohira's (1981) data. Low Q_{10} values (1.0 ~ 1.5) were reported for net ammonium uptake of low-temperature adapted ryegrass and oilseed rape (Clarkson and Warner, 1979; Macduff et al., 1987).

1.3.2.3. Long-term low temperature effects

The effect of root temperature on ion uptake varies with the treatment duration. Plants may adjust rates of ion transport in the long-term so that net uptake is independent of external variables such as temperature (Clarkson, 1976). As a result of plant adaptation to low root temperatures, NH_4^+ is absorbed more readily than NO_3^- at low temperatures by roots of Italian and perennial ryegrass (Lycklama, 1963; Clarkson and Warner, 1979; Clarkson et al., 1986) and lettuce (Frota and Tucker, 1972). Ammonium uptake by 4 day corn roots occurred even at temperatures as low as 0°C (Yoneyama et al., 1977).

In both *Indica* and *Japonica* rice plants ammonium and nitrate uptake and assimilation were strongly affected by temperature (Ta and Ohira, 1981). The uptake as well as assimilation of the two forms of nitrogen were greatly inhibited at low temperature and low light intensity. At low root temperature, uptake of NH_4^+ was higher than that of NO_3^-. The proportion of NH_4^+ absorbed from mixed NH_4^+ and NO_3^- solution was increased as root temperature decreased from 13 to 3°C (Macduff and Wild, 1989). Likewise, transferring corn roots from 30°C to 0°C, reduced

$^{15}NO_3^-$ uptake more drastically than $^{15}NH_4^+$ uptake (Yoneyama et al., 1977).

The lower sensitivity of NH_4^+ uptake to reduced temperature (compared to NO_3^- uptake) might be explained by a lesser dependence of NH_4^+ uptake on the rate of metabolism and energy production (Raven and Smith, 1976), or less effect of low temperature on enzymes of NH_4^+ assimilation (GS-GOGAT) compared to those enzymes of NO_3^- uptake and reduction (NR and NiR).

1.3.3. Effects of pH on NH_4^+ uptake

It has frequently been reported that NH_4^+ uptake is higher at elevated pH while NO_3^- uptake is stimulated at low pH (van den Honert and Hooysman, 1955; Fried et al., 1965; Jungk, 1970). When plants are grown in medium containing NH_4^+ as the solo source of N, the inevitable acidification of the medium may cause damage to the roots and even death of plants (Loo, 1931; Raven and Smith, 1976). Moreover root growth may be restricted in NH_4^+ medium even when the pH of the medium is controlled between 6.0 and 6.5 (Lewis et al., 1987).

1.3.3.1. Acidification of rhizosphere by NH_4^+ uptake

A major factor in N uptake is the change of rhizosphere pH associated with NH_4^+ uptake and its effect on plant growth, root morphology and capacity for ion uptake. It is well known that NH_4^+ uptake will cause acidification of the growth medium (Raven and Smith, 1976). At high NH_4^+ concentrations an enhanced NH_4^+ uptake by ectomycorrhizal fungi caused an accelerated medium acidification that indirectly inhibited growth

(Jongbloed and Borst-Pauwels, 1990). NH_4^+ has greater detrimental effects on plant roots than on shoots (Loo, 1931; Raven and Smith, 1976). Plants supplied with moderate concentrations of NH_4^+ generally grow poorly compared with plants supplied with other sources of nitrogen (Rufty et al., 1982b) or mixed NO_3^-/NH_4^+ supplies. Increased proportions of NH_4^+ in mixed NH_4^+ and NO_3^- nutrient solutions increased shoot:root ratios at all levels of root-zone pH (Vessey et al., 1990). When NH_4^+ and NO_3^- were supplied together, cumulative uptake of total nitrogen was not affected by pH or solution NH_4^+ : NO_3^- ratio (Raper et al., 1991b).

1.3.3.2. Retarded plant growth in acidic medium

Acidic growth medium will, in turn, affect plant growth and NH_4^+ uptake. Root growth was restricted by increased acidity between pH 6.0 to 4.0 (Arnon and Johnson, 1942; Islam et al., 1980). As the pH of the root-zone declined, therefore, NH_4^+ uptake decreased and NO_3^- uptake increased (Vessey et al., 1990). It was reported that the growth rate of soybean shoots and roots was reduced by increasing pH (Rufty et al., 1982b).

1.3.3.3. NH_4^+ toxicity and acidic damage

If acidification of the root medium is controlled, plant growth with NH_4^+ as the sole N source may be equal to growth with NO_3^- (Barker et al., 1966; Rufty et al., 1983; Tolly-Henry and Raper, 1986a, 1989; Findenegg, 1987; Vessey et al., 1990). Soybean plants can effectively utilize NH_4^+ as a nitrogen source as long as root-zone pH is strictly controlled and a balance is maintained between carbohydrate availability and acquisition of NH_4^+ (Rufty et al., 1983). It was suggested that the inhibition of plant growth at low pH was due to a decline in NH_4^+ uptake and a consequential limitation of growth by N stress (Vessey et al., 1990).

1.3.4. NH_4^+ fluxes at the plasma membrane

1.3.4.1. Net flux

NET FLUX (ϕ_{net}) describes the 'net' rate of ion uptake by roots. The net ion uptake from the medium (outside) into the cytoplasm is determined by the balance between influx and efflux. In practice, net flux

$$\phi_{net} = \phi_{oc} - \phi_{co} \qquad [2]$$

is measured by the disappearance of tested ion in the uptake solution.

1.3.4.2. Influx

INFLUX (ϕ_{oc}) is defined as the rate of inward movement of solute across a particular membrane. Strictly speaking influx should refer to the unidirectional movement measured during a very short period, short enough to discount the efflux. NH_4^+ influx is negatively correlated with plant N status in lower plants (Silver and Perry, 1981; Hartmann and Kleiner, 1982; Wiegel and Kleiner, 1982; Boussiba et al., 1984; Mazzucco and Benson, 1984; Rai et al., 1984; Jayakumar et al., 1985), and higher plants (McCarthy and Goldman, 1979; Pelley and Bannister, 1979; Smith, 1982; Ullrich et al. 1984; Holtel and Kleiner, 1985; Clarkson, 1986; Lee and Rudge, 1986; Morgan and Jackson, 1988a, 1988b; Clarkson and Lüttge, 1991). MA influxes of pea seedlings decreased after pretreatment with glutamine and NH_4^+ and increased after pretreatment with asparagine (Deane-Drummond, 1986).

1.3.4.3. Efflux

EFFLUX (ϕ_{co}) is the rate of outward solute flow from cytoplasm across the plasma membrane. Efflux of ions from plant roots was identified in

plants under stress or damaged conditions (Pitman, 1963; Hope et al., 1966; Jackson and Edwards, 1966; Hiatt and Lowe, 1967; Ayers and Thornton, 1968; Bowen, 1968). In intact plants, efflux of K^+, Na^+, $H_2PO_4^-$, Cl^-, Br^-, or NO_3^- has been observed from roots (MacRobbie, 1964; Cram, 1968, 1973; Dodd et al., 1966; Poole, 1969, 1971a, 1971b; Pitman, 1971; Morgan et al., 1973; Macklon, 1975a, 1975b; Macklon and Sim, 1976, 1981; Behl and Jeschke, 1982; Jeschke, 1982; Lazof and Cheeseman, 1986; Siddiqi et al., 1991).

Continuous NH_4^+ efflux may be a common feature of net NH_4^+ uptake by roots of higher plants (Morgan and Jackson, 1973). In a study using intact ryegrass, $^{14}NO_3^-$-grown roots were equilibrated in a $^{15}NO_3^-$ solution enriched with ^{15}N (97.5 atmo %). The results suggested that there was a simultaneous occurrence of the influx of $^{15}NO_3^-$ and efflux of $^{14}NO_3^-$ (Morgan et al., 1973). Moreover, careful measurements of $^{14}NH_4^+$ efflux revealed that there must have been generation of NH_4^+ by breakdown of nitrogen compounds during the course of the experiment. There was excess quantity of $^{14}NH_4^+$ effluxes compared with the initial content in the roots (Morgan and Jackson, 1988a). There is even an $^{14}NH_4^+$ efflux from $^{14}NO_3^-$-grown roots (Morgan and Jackson, 1988b).

1.3.4.4. Balance of fluxes

There is thought to be an ammonium cycle across the root cell plasma membrane (Morgan and Jackson, 1988b). It was reported that endogenous NO_3^- effluxes to the unstirred layers were recycled through NO_3^- influx (Morgan et al., 1973). The same could be expected for NH_4^+ efflux. Substantial ammonium cycling occurred during net ammonium uptake (Jackson et al., 1993), yet plants grown under low N conditions possess a low NH_4^+ efflux. Morgan and Jackson (1988a) suggested that the

regulation of NH_4^+ uptake by roots of higher plants may involve changes of both influx and efflux in response to plant nitrogen status. It was found that net $^{15}NH_4^+$ influx was increased and net $^{14}NH_4^+$ efflux was decreased in nitrogen depleted wheat and oat seedlings (Morgan and Jackson, 1988a), and net NH_4^+ uptake of barley and maize plants previously grown with NH_4^+ was decreased subsequently (Morgan and Jackson, 1988b).

The determining factor may be the internal $[NH_4^+]$ of the root cell. For example, enhanced NH_4^+ influx by MSX treatment was claimed to be due to the enlargement of cytoplasmic and vacuolar NH_4^+ pools of root tissue several times (Jackson et al., 1993; Lee and Ayling, 1993) which appeared enhance the influx of $^{13}NH_4^+$ of (maize and barley) plants by reducing isotopic efflux (Lee et al., 1992; Lee and Ayling, 1993). However, the enlarged $[NH_4^+]_i$ was also advanced to explain the enhanced efflux observed in their system (Morgan and Jackson, 1988b).

1.3.4.5. N cycling in the whole plant

Within the plant, N cycling, the simultaneous movement of N-compounds from root to shoot, and from shoot to root (Cooper and Clarkson, 1989; Larsson et al., 1991) may enable N absorption to be regulated to match the demand imposed by plant growth (Drew and Saker, 1975; Edwards and Barber, 1976). The concentrations of amides (Gln and Asn) in the roots will be the result of the balance between their synthesis from absorbed inorganic N (NH_4^+ or NO_3^-), their import via the phloem, and their export via the xylem (Lee et al., 1992).

1.3.5. Regulation of ammonium uptake

Feedback inhibition of NH_4^+ uptake by nitrogenous effectors has been implicated in lower plants (Kleiner, 1985; Ullrich et al., 1984; Pelley and Bannister, 1979; MacFarlane and Smith, 1982; Wiame et al., 1985; Wright and Syrett, 1983; Thomas and Harrison, 1985) and higher plants (Cook and Anthony, 1978b; Breteler and Siegerist, 1984; Wiame et al., 1985; Revilla et al., 1986; Lee and Rudge, 1986; Morgan and Jackson, 1988a). There is, however, only limited information available concerning the possible mechanism(s) of regulating NH_4^+ uptake by either NH_4^+ *per se* or its primary assimilates.

1.3.5.1. Negative feedback regulation

At high nitrogen status, plant NH_4^+ uptake could be suppressed due to (i) low energy supply to the root system, (ii) accumulation in the root tissue of nitrogenous compounds that exerts negative feedback on the transport system, or (iii) high efflux of endogenous NH_4^+ (Morgan and Jackson, 1988b). Repression of NH_4^+ uptake may be due to continual generation of ammonium from degradation of organic nitrogenous sources within roots and rapid accumulation of ammonium in roots of N-depleted plants upon initial exposure to ammonium (Morgan and Jackson, 1988a, 1988b). However, Morgan and Jackson (1988b) indicated that the immediate assimilates of NH_4^+, such as glutamine, are more likely negative effectors on NH_4^+ uptake.

1.3.5.2. Enhanced NH_4^+ uptake

Negative correlation between ammonium uptake and cell nitrogen status have commonly been observed (McCarthy and Goldman, 1979; Pelley and Bannister, 1979; Smith, 1982; Ullrich et al. 1984; Holtel and

Kleiner, 1985; Clarkson, 1986; Lee and Rudge, 1986; Morgan and Jackson, 1988a, 1988b; Clarkson and Lüttge, 1991). It has been recognized that the capacity for nitrogen uptake is enhanced in N-depleted plants such as wheat (Tromp, 1962; Minotti et al., 1969; Jackson et al., 1976b; Morgan and Jackson, 1988a, 1988b); ryegrass (Lycklama, 1963); maize (Ivanko and Ingversenm, 1971; Lee et al., 1992); barley (Lee and Rudge, 1986); and oats (Morgan and Jackson, 1988a, 1988b).

1.3.6. Interactions between NH_4^+ and K^+

1.3.6.1. Mutual beneficial effects between N and K

N and K are essential plant nutrients, required for healthy plant growth and high yield production (Ajayi et al., 1970; Dibb and Welch, 1976; Kemmler, 1983; Dibb and Thompson, 1985; Grist, 1986; Biswas et al., 1987; Dey and Rao, 1989; Ichii and Tsumura, 1989; Fageria et al., 1990; Xu et al., 1992). Mutual beneficial effects of K and N on plant growth have often been described. An adequate K^+ supply has been shown to enhance NH_4^+ uptake and assimilation (Ajayi et al., 1970; Barker and Lachman, 1986; Scherer and MacKown, 1987). Sufficient N nutrition normally promotes K^+ uptake due to the biological dilution effect of better plant growth (Noguchi and Sugawara, 1966; Kirkby, 1968; Claassen and Wilcox, 1974; Faizy, 1979; Lamond, 1979; Beusichem and Neeteson, 1982).

1.3.6.2. Inhibition of K^+ uptake by NH_4^+

However, NH_4^+ has been shown to strongly inhibit the absorption of K^+ in short-term experiments in many species including wheat, barley, maize and tobacco (Breteler, 1977; Munn and Jackson, 1978; Rufty et al.,

1982; Rosen and Carlson, 1984; Scherer et al., 1984). There was a negative correlation between the external NH_4^+ concentrations and K^+ uptake (Rosen and Carlson, 1984; Scherer et al., 1987; Jongbloed et al., 1991), and net ammonium uptake was correlated with potassium efflux (Morgan and Jackson, 1989).

The inhibitory effect of NH_4^+ on K^+ uptake has been claimed to be independent of K^+ provision or pretreatments; it is probably exerted on the transport processes at the plasma membrane. Insufficient evidence is available to draw a conclusion regarding the inhibition of K^+ uptake by NH_4^+ in terms of competitive and non-competitive effects (Deane-Drummond and Glass, 1983b; Scherer et al., 1984). K^+ uptake was suppressed during rapid NH_4^+ uptake by N-starved plants (Tromp, 1962), but K-starvation did not produce the same effect as N-starvation on the transport of NH_4^+ (Tromp, 1962; Lee and Rudge, 1986).

1.3.6.3. Inhibition of NH_4^+ uptake by K^+

On the other hand, NH_4^+ uptake of plants was not reduced by K^+ in the nutrient medium (Mengel et al., 197 6; Rosen and Carlson, 1984; Scherer and Mackown, 1987). However, the influence of K^+ on NH_4^+ uptake has not been consistent. It was reported that K^+ had inhibitory effects but did not compete with NH_4^+ for selective binding sites in the absorption process (Ajayi et al., 1970; Dibb and Welch, 1976; Mengel et al., 1976).

1.4. Research Objectives

The objective of this study was to investigate the mechanisms and characteristics of ammonium uptake by rice plants. In particular, the

studies have emphasized short-term responses of fluxes to changes in ambient conditions. This particular goal was achieved by using the short-lived radioisotope ^{13}N ($t_{1/2}$ = 9.98 min), addressing five different areas:

(1). By measuring NH_4^+ influx and efflux, the exchange of N at the plasma membrane and the relationships between these fluxes were quantified. Subcellular distribution of absorbed NH_4^+ was also estimated. The results of these studies are interpreted in terms of a root cell model in Chapter 3.

(2). To describe the kinetics of NH_4^+ uptake and the pattern(s) of its concentration dependence, NH_4^+ influx was measured in perturbation experiments in plants grown in different levels of N. By altering ambient conditions such as medium pH, root temperature, and by treating roots with various metabolic inhibitors, the energetic of NH_4^+ uptake was investigated. These are described in Chapter 4.

(3). By measuring electrical potential differences together with assaying cytoplasmic [NH_4^+], the electrochemical potential gradient for NH_4^+ between external solution and cytosol were defined in order to explore the mechanisms of NH_4^+ uptake. Membrane electrical potential differences of rice roots were recorded as a function of external NH_4^+ concentration. This information is incorporated with data dealing with biochemical, kinetic and energetic aspects of NH_4^+ uptake to formulate a model for the mechanisms of NH_4^+ uptake (Chapter 5).

(4). Without information on the regulation of NH_4^+ uptake, the uptake model is incomplete. NH_4^+ influx was measured as a function of root N status. Internal [NH_4^+] was determined as well as the concentrations of individual amino acids. In Chapter 6, the results are discussed in reference to existing reports to develop a model of the regulation of NH_4^+ uptake.

(5). Chapter 7 deals with the interactions between NH_4^+ and K^+ at the uptake level and explores the effects of prior exposure to these ions on subsequent ion uptake.

Chapter 2. METHODS AND MATERIALS

In this chapter, the general methods used in this study are described. Method(s) used in a particular experiment will be addressed in the corresponding chapter.

2.1. PLANT GROWTH

2.1.1. Seed germination

Rice seeds (*Oryza sativa* L. cv. M202) were surface sterilized in 1% NaOCl for 30 min and rinsed several times with de-ionized distilled water. Seeds were imbibed overnight in aerated de-ionized distilled water at 38°C, then placed on plastic mesh mounted on Plexiglas discs. The discs were set in a Plexiglas tray filled with de-ionized distilled water just above the level of the seeds, and seeds were allowed to germinate in a growth chamber in the dark (at 38°C) for 4 d. During the following 2 d, the temperature was stepped down to 20°C (by 9°C per day). Then discs, with one-week-old rice seedlings, were transferred to 40-L Plexiglas tanks.

2.1.2. Growth conditions

Plants were grown hydroponically in 40-L Plexiglas tanks located in a walk-in growth room, in which growth conditions were maintained as follows: temperature: 20 ± 2°C; relative humidity: 75%; and irradiance: 300 $\mu E\ m^{-2}\ s^{-1}$ under fluorescent light-tubes (VITA LITE, Duro-Test) on a cycle

of 16 h light and 8 h dark. Plants were 3-week-old when they were used for most experiments unless specifically indicated.

2.1.3. Provision of nutrients

The growth medium was modified based on the recipe of modified Johnson's nutrient solution (Johnson et al., 1957; Epstein, 1972) and a recipe from the International Rice Research Institute (Yoshida et al., 1972), in which ammonium (NH_4Cl) was the only source of nitrogen (except for some specific experiments as specifically indicated) and silicon was added as $Na_2SiO_3.5H_2O$. This modified Johnson's nutrient solution (hereafter referred to as **MJNS**) was also the medium used to carry out all experiments. The composition of this MJNS, in micromolar (μM), was 200 for Ca, K and P, 100 for Mg, 300 for S, 16 for B, 5 for Si and Fe, 1 for Mn and Zn, 0.3 for Cu and Mo. The external ammonium concentration ($[NH_4^+]_o$) was varied as indicated at the appropriate places. Generally plants were grown in MJNS containing 2, 100, or 1000 μM $[NH_4^+]_o$, referred to hereafter as G2, G100, G1000 plants, respectively. The concentrations of nutrients in growth medium were maintained by infusion of appropriate stock solutions, through peristaltic pumps (Technicon Proportioning Pump II, Technicon Inst. Corp.). Generally 2 liters per day of stock solution were supplied and stock concentrations were determined from daily chemical analyses of medium samples. Solutions were mixed continuously by circulating pumps (Circulator Model IC-2, Brinkmann Inst., Inc.), and aerated continuously. The pH of growth medium was maintained at 6.0 ± 0.5 by adding powdered $CaCO_3$ (1~3 g/tank), according to measured pH values, 1~2 times daily.

2.2. N ISOTOPES FOR STUDYING N UPTAKE

2.2.1. Isotopic tracer

There is now widespread use of isotopic tracers, particular radioactive tracers, in the biological sciences (Thain, 1984). Carbon (^{11}C, ^{14}C), phosphorus (^{32}P), sulfur (^{35}S), chlorine (^{36}Cl), potassium (^{42}K), rubidium (^{86}Rb), calcium (^{45}Ca) and sodium (^{22}Na) have been employed to determine the kinetics of transport and transformation of these elements in living systems. Measurements of radioisotopic influx and/or efflux have been used to obtain an estimate of the unidirectional fluxes of the stable isotope of the ion at the plasmalemma and tonoplast and to estimate the separate amounts of the stable isotopes in the cytoplasm and vacuole (Cooper, 1977; Thain, 1984).

The utility of radiochemical techniques is afforded by (i) their great sensitivity compared to other analytical methods. Radioisotopic tracers may offer 10^8-fold increased detection sensitivity over stable isotope methods (Cooper, 1977; Krohn and Mathis, 1981); (ii) the fact that they "label" the atoms of molecules without significantly altering their chemical properties (Cooper, 1977; Boyer, 1986).

2.2.2. Nitrogen Isotopes

There are six isotopes of nitrogen known, ranging in mass number from 12 to 17 (Kamen, 1957). The stable isotopes of nitrogen are ^{14}N and ^{15}N, the latter being present to the extent of 0.365 atom per cent. Radioactive isotopes ^{12}N and ^{13}N are positron emitters with half-lives of

0.0125 seconds and 9.98 minutes respectively. ^{16}N and ^{17}N are negatron emitters with half-lives of 7.35 and 4.14 seconds respectively, ^{17}N also emits neutrons. The longest-lived radioactive isotope of nitrogen is ^{13}N which is the only radioactive isotope that has been used in tracer research (Kamen, 1957; Krohn and Mathis, 1981; Bremner and Hauck, 1982). The use of ^{15}N (Burris and Miller, 1941) in biological studies started as early as the use of ^{13}N (Ruben et al., 1940).

2.2.3. Stable ^{15}N techniques

Since the first use of ^{15}N (Burris and Miller, 1941), this isotope has been widely used in agricultural research (Hauck, 1982; Knowles and Blackburn, 1993), and the analytical methodology has been continuously improved (Clusius and Backer, 1947; Hoch and Weisser, 1950; Hürzeler and Hostettler, 1955; Broida and Chapmen, 1958; Faust, 1960; Mulvaney and Liu, 1991; Hoult et al., 1992).

^{15}N has been used in characterizing the NO_3^- and NH_4^+ uptake processes of plants (Fried et al., 1965; Yoneyama and Kaneko, 1989; Yoneyama et al., 1991) and tracing the metabolism of nitrogen in plant cells (Yoneyama and Kumazawa, 1975; Arima and Kumazawa, 1977). ^{15}N is also widely used in studying N_2-fixation in soil-plant systems, aquatic and sediment systems (Watanabe, 1993; Warembourg, 1993) and N transformation in soils (Azam et al., 1993). It is also employed in studying the mineralization of soil organic N (Powlson and Barraclough, 1993) and nitrification and denitrification of soil N (Mosier and Schimel, 1993). ^{15}N-labeled nitrogen fertilizer has also been used in the study of fertilizer use efficiency (Azam et al., 1991).

Stable N isotope techniques have several advantages over techniques using radionuclides. As a biochemical tracer, ^{15}N offers the advantages of being relatively inexpensive, widely available, free of radiation hazard and less limiting in terms of experiment duration. The advantages of using ^{15}N also embodies a major disadvantage in its use as a tracer: a sizable background, present in all nitrogenous materials, against which added tracer must be measured (Cooper et al., 1985). In order to measure significant enrichment of ^{15}N in specific metabolic compartments, investigators have to administer a large amount of ^{15}N-labeled nonphysiological precursors to biological systems (Cooper et al., 1985). In addition it requires tedious preparation to convert samples to N_2 gas prior to mass or emission spectrometry.

2.2.4. Radioactive isotope, ^{13}N

2.2.4.1. Use in biological studies

^{13}N was first made in 1934 by Joliot and Curie as $^{13}NH_4^+$ and was one of three isotopes generated artificially by induction of radioactivity in otherwise stable elements (boron) by bombardment with particles emitted by polonium (Joliot and Curie, 1934). It was first used as a biological tracer in studying the N_2-fixation of non-legume barley plants (Ruben et al., 1940), which was one year earlier than the first report of using $^{15}N_2$ to study N_2 fixation (Burris and Miller, 1941).

Much of the early tracer work in biochemistry was carried out with positron-emitting radionuclides, such as ^{11}C, and to a lesser extent ^{13}N, but with the introduction of ^{13}C, ^{14}C and ^{15}N, their importance declined over a period of two decades. Only in the past 10 years or so, have these short-

lived isotopes again become important as tracers particularly in the field of biochemical research. With about 70 medical cyclotrons, there are at least 12 groups, that generate ^{13}N for biological studies (Cooper et al., 1985). In biological studies, there are several groups using ^{13}N in study nitrogen nutrition of plants (**Appendix A**).

2.2.4.2. Production of ^{13}N

^{13}N can be obtained from targets containing boron, carbon, nitrogen or oxygen and an appropriate accelerated particle (Cooper et al., 1985). The $^{10}B(\alpha,n)^{13}N$; $^{12}C(d,n)^{13}N$; $^{12}C(p,\gamma)^{13}N$; $^{13}C(p,n)^{13}N$; $^{14}N(p,pn)^{13}N$; $^{14}N(n,2n)^{13}N$ and $^{16}O(p,\alpha)^{13}N$ reactions have all been used to make ^{13}N (Krohn and Mathis, 1981; Tilbury, 1981). The method most widely used at present for the production of ^{13}N-ammonia is the proton irradiation of water ($^{16}O(p,\alpha)^{13}N$), followed by reduction of the $^{13}NO_3^-$ and $^{13}NO_2^-$ formed under typical conditions of irradiation (Park and Krohn, 1978; McElfresh et al., 1979; Lindner et al., 1979; Tiedje et al., 1979; Chasko and Thayer, 1981; Cooper et al., 1985).

An example flow scheme of $^{13}N_2$ production based on nuclear reactions of $^{16}O(p,\alpha)^{13}N$ is as follows: (Meeks et al.,1985)

1. Generation: $H_2O \xrightarrow{\text{20 MeV, 20 } \mu A} {}^{13}NO_3^- + {}^{13}NO_2^- + {}^{13}NH_4^+$

2. Concentration: 60 ml $^{13}NO_3^-$ $\xrightarrow{\text{HPLC/SAX column}}$ up to 3 ml $^{13}NO_3^-$

3. Reduction: $^{13}NO_3^- \xrightarrow[\text{65°C Saturated NaOH}]{\text{Devarda's Alloy (Cu/Al/ Zn)}} {}^{13}NH_3$

4. Trapping: $^{13}NH_3 + H^+ \longrightarrow {}^{13}NH_4^+$

5. Oxidation: $^{13}NH_4^+$ $\xrightarrow[1.5\ \mu mol\ ^{14}NH_4^+]{Na/KOBr}$ $[^{13}N]\text{-}N_2$

The yield of ^{13}N varies with the types of nuclear reaction, target material, and particle energy. Bombarding 10 ml pure water with an 10 μA proton beam of high energy (>19 MeV) could yield 36 mCi μA^{-1} 20 min^{-1} (Vaalburg et al., 1975). The ^{13}N species, $^{13}NO_3^-$, $^{13}NO_2^-$ and $^{13}NH_4^+$, are present in the radioactive sample. The relative concentrations of these species is dependent upon the irradiation dose as well as on other factors such as the previous irradiation history of the target foil (Tilbury and Dahl, 1979). The study of the effect of integrated dose showed that at low dose $^{13}NH_4^+$ is greater than $^{13}NO_2^-$ and at high dose $^{13}NH_4^+$ is less than $^{13}NO_2^-$ (Tilbury and Dahl, 1979).

There are also some contaminants in the radioactive product. It was found that irradiated unprocessed water contains ^{18}F ($t_{1/2}$=1.8 h), ^{15}O ($t_{1/2}$=2.0 min), and ^{48}V ($t_{1/2}$=16.2 d). Both ^{18}F and ^{48}V produce no problems with $^{13}NH_4^+$ since these radioisotopes do not distil. Since ^{18}F is from the reaction of $^{18}O(p,n)^{18}F$, its contamination can be minimized by depleting ^{18}O in water (Skokut et al., 1978). Though ^{15}O can be detected in ^{13}N-ammonia solution, it will disappear during preparations lasting more than 20 min (Vaalburg et al., 1975).

The ^{13}N isotope disintegrates by emission of a positron (β^+, 1.2 MeV of maximum emission energy) giving rise to ^{13}C (Meeks, 1993). In annihilation reaction between a positron and an electron, two gamma photons are formed each of 0.511 MeV energy traveling in nearly opposite directions (Cooper et al., 1985; Meeks, 1993). Therefore the detection of radioactive decay in the sample is accomplished in a gamma counter. Since ^{13}N decay results in Cerenkov light, it may be counted by the

photomultiplier tubes in liquid scintillation systems (Glass et al., 1985). Radioactivity is typically counted immediately in a gamma counter and all counts are decay-corrected to a common time. The admitted ^{13}N in plant tissues can be observed by placement of multiple Gieger-Mueller tubes along the plant axis (McNaughton and Presland, 1983; Caldwell et al., 1984), by autoradiography on X-ray film between blocks of dry ice for 20 - 30 min (Deane-Drummond and Thayer, 1986), or by hand-sectioning of the tissue and scintillating counting.

2.2.4.3. Advantages of the use of ^{13}N in biological studies

The use of $^{13}NH_4^+$-N in biological studies of nitrogen nutrition has several advantages:

(1) The chief advantage is that such nuclide can be prepared at a very high specific activity increasing sensitivity for detection approximately 10^8-fold, to trace rapid kinetics and metabolic pathways (Krohn and Mathis 1985). Because of the great sensitivity of the radioactive isotope technique, ^{13}N has proved to be of value in elucidating biological mechanism over very short time intervals. (Hanck, 1982).

(2) In order to measure the initial events in biological processes it may be necessary to determine events on a time scale of seconds to minutes. High specific activity tracers which are detected with high efficiency (e.g. ^{13}N) make possible such measurements. It is clear that time resolution of a tracer-influx experiment is crucial for subsequent interpretation of the fluxes. In short term experiment, by using $^{13}NO_3^-$, one is able to monitor net uptake and disappearance of $^{13}NO_3^-$ simultaneously, thus increasing the experimental resolution compared with experiments where plants

have to be sampled and further prepared before assay (Oscarson et al., 1987).

(3) The isotope decays rapidly ($t_{1/2}$ = 9.98 min). After allowing sufficient time for decay, repeat studies can be carried out in the same system without interference from previously administered tracer (Cooper et al., 1985). In tissue dissection or *in vitro* studies, the total quantity of tracer present in rather large specimens can be determined rapidly and accurately, with little sample preparation, by gamma counting techniques.

(4) ^{13}N is inherently less hazardous to use in comparison with conventional, much longer lived tracers. The problem of radioactive waste disposal is eliminated (Cooper et al., 1985).

Nevertheless, the disadvantages are also related to its short half-life. It is only available at relatively few research centers located close to the cyclotron. Its production requires a suitable accelerator and a correspondingly large capital investment (Cooper et al., 1985). Its short half-life limits the period over which it can be used to a maximum of perhaps 4 hours or so depending on the application (Meeks, 1993). Techniques of precursor synthesis, labeling, product purification, metabolic separation and analysis must be appropriately rapid (Fuhrman et al., 1988).

2.2.4.4. Considerations of using ^{13}N in nitrogen uptake

To study nitrogen uptake, especially ammonium uptake by plant roots, several facts have to be considered:

(1) Membrane fluxes of nitrogen are of utmost importance for the over-all nitrogen utilization in plant growth.

(2) Ammonium is rapidly metabolized to amino acids and amides within the root before transport to the shoot (Pate, 1973). Evidence showed that the NH_4^+ uptake rate is also regulated by the N assimilation and translocation rates of the plants (Wiame et al., 1985; Morgan and Jackson, 1988). Therefore it is necessary to identify the nitrogen compounds in the uptake, assimilation and transport processes.

(3) It is difficult to measure the subcellular, i.e. cytoplasmic and vacuolar, pools of NO_3^- and/or NH_4^+ directly due to their small size and rapid turn-over. It was found that the half-time for exchange of the cytoplastic NO_3^- pool ranged from 2 to 5 minutes in roots of *Zea Mays* (McNaughton and Presland, 1983).

(4) Ion uptake of plant roots is able to adapt during a long-term experiments in response to changes of environmental conditions, such as temperature or pH (Macduff et al., 1987). Therefore the tracer technique can be chosen as a proper approach to study ammonium uptake by rice roots in consideration of high sensitivity, rapid measurement and short duration of experiments. Another point is that uptake by depletion is so slow from high external concentration that it can not be measured except with ^{13}N.

2.2.4.5. Use of ^{13}N in nitrogen transport studies

In short-term experiments, ^{13}N has been used to study nitrogen uptake by plant roots (McNaughton et al., 1983; Glass et al., 1985; Lee et al., 1986; Oscarson et al., 1987). Most reported studies used $^{13}NO_3^-$ in uptake experiments; few made use of $^{13}NH_4^+$. $^{13}NO_3^-$ has been used to identify and characterize the transport systems (Thayer and Huffaker, 1982; McNaughton and Presland, 1983; Siddiqi et al., 1990; Glass et al.,

1990); regulation of influx (Glass et al., 1985; Oscarson et al., 1987; Siddiqi et al., 1989; Rufty et al., 1991); and cell compartmentation (Presland and McNaughton, 1984; Lee et al., 1986; Siddiqi et al., 1991). Presland et al., (1986) were able to use $^{13}NH_4^+$ to study ammonium uptake by roots of hydroponically grown maize seedlings and the transport of ^{13}N to the shoot. It was found that the rate of uptake of ammonium, by *Zea mays*, was a function of external ammonium ion concentration at less than 1 mM.

2.2.4.6. Use of ^{13}N in nitrogen assimilation

^{13}N has also proven useful in understanding nitrogen assimilation in plant cells. Gln is the first major organic product of $^{13}NH_4^+$ assimilation (Skokout et al., 1978) and the GS/GOGAT pathway is the primary route of assimilating fixed ^{13}N (Meeks et al., 1978a). It was found that MSX inhibited the incorporation of $^{13}NH_4^+$ into Gln more than into Glu. The opposite was true for $^{13}NO_3^-$. In tobacco cells GDH only plays a minor role (Skokout et al., 1978) but in non-leguminous angiosperm N_2-fixers, GDH may play a major role in the assimilation of exogenously supplied NH_4^+ (Schubert et al., 1981).

Since $^{13}NH_4^+$ can be produced in hundreds of millicuries, it should be possible to synthesize a large number of ^{13}N-labeled amino acids, nucleotides, amino sugars, and other metabolites via known enzymatic routes (Cooper et al., 1985). Organic N-containing compounds, such as L-(^{13}N)-glutamate and L-(amide-^{13}N)-glutamine, are also synthesized from $^{13}NH_4^+$ and used in studies of NH_4^+ and glutamine assimilation pathways (Suzuki et al., 1983; Calderón et al., 1989). It was found in *Neurospora crassa* that (^{13}N)-Gln is metabolized to (^{13}N)-Glu by GOGAT and to $^{13}NH_4^+$ by the glutamine transaminase-ω-amidase pathway. Then released $^{13}NH_4^+$ is reassimilated by both GDH and GS (Calderón et al., 1989). Extracted ^{13}N-

labeled amino acids or amides can be separated by HPLC and electrophoresis (Cooper et al., 1979; Meeks, 1993). It was found that translocation of N compounds can also be traced by ^{13}N. Barley leaves exposed to $^{13}NH_3$ gas for 30 min, incorporated ^{13}N mainly into free Gln and Glu and 1 to 3% of these were exported to the sheaths through the phloem (Hanson et al., 1979).

2.2.4.5. Use of ^{13}N in denitrification

In addition ^{13}N has also been used to study denitrification in soils (Gersberg et al., 1976; Tiedje et al., 1979; Bremner and Hauck, 1982). Use of ^{13}N allows the direct quantitative measurements of denitrification rates over short time intervals, without changing the concentration of NO_3^- in the soil system from flooded rice fields (Gersberg et al., 1976).

2.2.5. Protocol for $^{13}NH_4^+$ production in present study

The short-lived radioisotope ^{13}N ($t_{1/2}$ = 9.98 minutes) was produced as described by Siddiqi et al., (1989), by 20 MeV-proton irradiation of H_2O on an ACEL CP42 cyclotron. Contaminants in the $^{13}NO_3^-$ sample (mainly ^{18}F) were removed by passing the samples twice through a SEP-PAC Alumina-N cartridge (Waters Associates). Reduction of $^{13}NO_3^-$ to $^{13}NH_3$ was achieved by using Devarda's alloy at 70°C in a water bath (Vaalburg et al., 1975; Meeks et al., 1978); $^{13}NH_3$ was separated from remaining chemical species by distillation at alkaline pH, and trapping in acid solution as $^{13}NH_4^+$. The flow scheme for this conversion is shown in Figure 1.

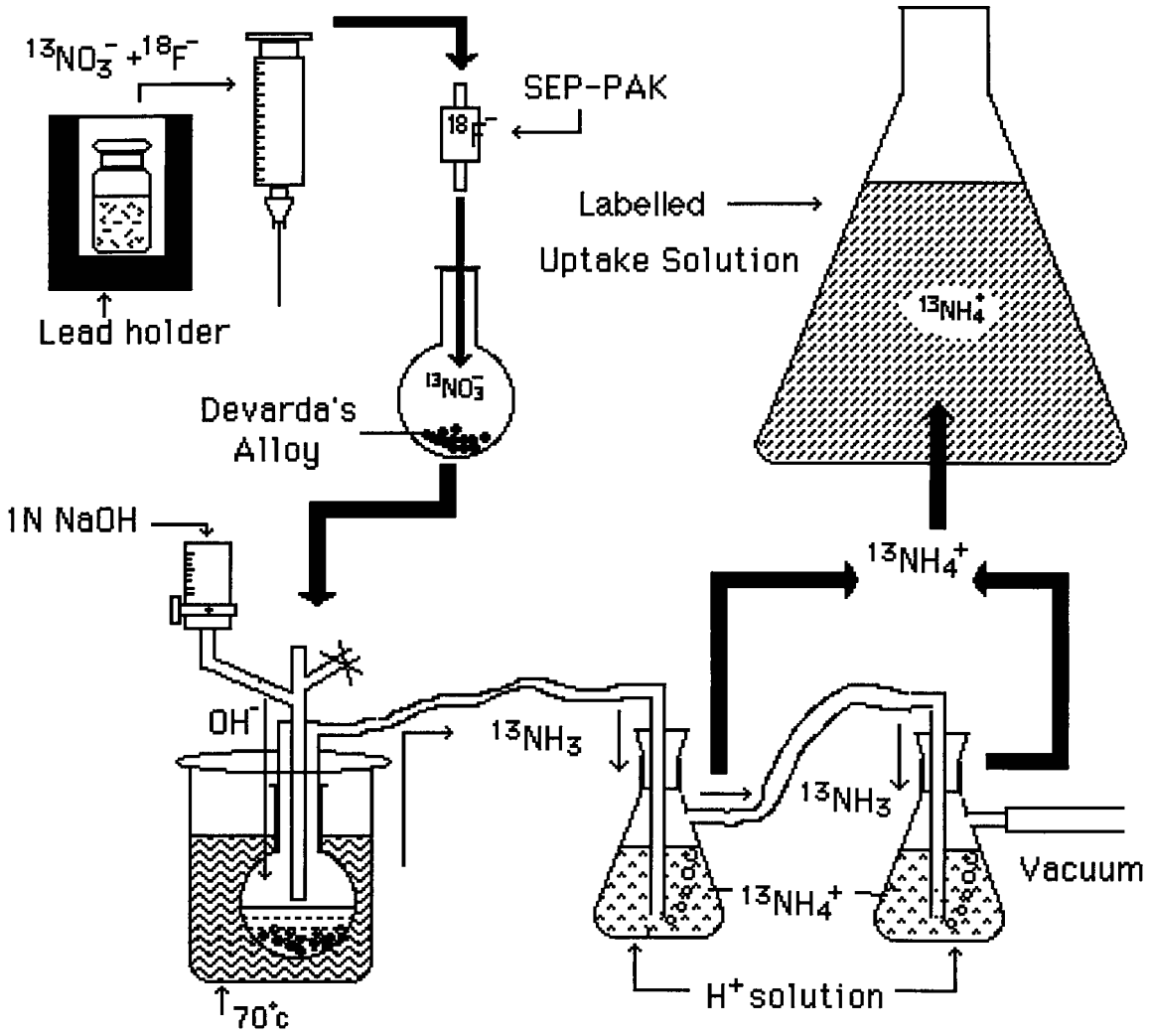


Figure 1. The flow scheme for $^{13}NH_4^+$ production. (As described in Section 2.2.5.)

2.3. MEASUREMENT OF NH_4^+ FLUXES

2.3.1. Influx of $^{13}NH_4^+$

Standard procedures for $^{13}NH_4^+$ uptake were as follows: (a) loading: rice roots were loaded in $^{13}NH_4^+$-labeled MJNS (hereafter referred to as 'loading' solution) for designated periods; (b) pre-wash and post-wash: prior to and after loading, roots were pre-washed and post-washed in un-labeled MJNS (hereafter referred to as 'washing' solution) for 5 min and 3 min, respectively. The choice of these times is rationalized in the Discussion section (section 3.4.). Experiments were conducted at steady-state with respect to $[NH_4^+]_o$, i.e., the $[NH_4^+]_o$ of 'washing' solutions and 'loading' solutions were the same as those provided during the growth period or in experiments to define influx isotherms; plants were exposed to different $[NH_4^+]_o$ for short (perturbation) experiments. Immediately after the post-wash period, plants were cut into shoots and roots and the surface liquid adhering to the roots was removed by a standard 30 sec spin in a slow-speed table centrifuge (International Chemical Equipment, Boston). Roots and shoots were introduced into separate scintillation vials and immediately counted in a gamma counter (MINAXI γ-5000, Packard). The fresh weights of roots and shoots were recorded immediately after counting.

2.3.2. Efflux of $^{13}NH_4^+$

Roots of rice seedlings were immersed in the $^{13}NH_4^+$ labeled 'loading' solution for 30 min. At the end of this time plants were transferred to an

elusion vessel and tracer leaving the roots in exchange for $^{14}NH_4^+$ in the un-labeled identical 'washing' solution. This solution was collected at prescribed interval in 20-ml scintillation vials for counting.

2.3.3. Net flux of NH_4^+

Net NH_4^+ flux was measured in uptake solutions by the depletion method. Solution samples (S_1 and S_2) were taken at different times (t_1 and t_2), and the difference of assayed [NH_4^+] was used to calculate net NH_4^+ flux. Net NH_4^+ flux can also be estimated by subtracting efflux from influx of the same roots.

2.4. COMPARTMENTAL (EFFLUX) ANALYSIS

2.4.1. Compartmentation of plant cells

Plant cells are highly compartmentalized. They are surrounded by the cell wall, and the plasma membrane encloses the cytoplasm, in which are found the vacuole, mitochondria, nucleus, plastids and other organelles. Up to 80% or more of cell volume is occupied by the vacuole which is enclosed by the tonoplast (Salisbury and Ross, 1985). The cytoplasm is the vital part of cell. The major functions of the vacuole are to maintain turgor which contributes to cell shape and to store solutes. The compartmentation of the cell has important consequences for nutrient uptake, unidirectional fluxes, assimilation, distribution and translocation. Because higher plant cells are too small to dissect and the size of the compartments is even

smaller, it seems technically impossible to obtain information on the composition of each compartment. However, through various methods, such as NMR, ion-specific electrodes, EDX, compartmental analysis, or fluorescent dyes, the ion concentration of one or more particular compartments, or fluxes between compartments can be estimated. Compartmental analysis is the only systematic method of investigating transport processes and estimating the size of compartments and to analyze the kinetics of movement of ions to or from a tissue (Cram, 1968). Therefore it has been established as a tool for characterizing the exchange properties of multicompartment systems.

2.4.2. Development of theory

Compartmental analysis was first used by Fourier in 1822 to describe the relationships between heat flow and temperature gradients and, in 1855, it was adopted by the biologist, Fick, in studying diffusive flow along a concentration gradient (Zierler, 1981). Not until one century later, was it introduced by MacRobbie and Dainty (1958) to study ion transport in *Nitellopsis*. Soon after, Pitman (1963) was the first to use this method to investigate multicompartmental transport processes in a higher plant. Compartmental analysis has mostly been used by plant physiologists to calculate the fluxes, characterize internal ion pool sizes and membrane kinetic parameters for ion exchange.

The basic assumption of this methodology is that the system is at steady state, or at equilibrium. Additional assumptions include that (1) the substance of interest flows into and from the separate compartments of the system; (2) the flux is proportional to the quantity (or concentration) of

the substance in the compartment from which the material flows. It is assumed that the material under study is neither destroyed nor synthesized in any compartment, and that each compartment is homogeneous, or well stirred; (3) the concentration of an ion species or its flux is described by a first-order linear differential equation with constant coefficients which are independent of elapsed time and of the conjugate (Zierler, 1981). For higher plant systems, the additional assumption is that the relevant compartments of the experimental system are functionally in series with each other (Walker and Pitman, 1976; Cheeseman, 1986). These assumptions may not always be valid (Lazof and Cheeseman, 1986). It is suggested that compartmental efflux analysis should not be used alone, but integrated with other methods such as influx measurements (Cheeseman, 1986).

2.4.3. Models for compartmental analysis

The testing model or the analysis process can be varied with the research subject (excised tissue or intact plant), number of compartments (2, 3, or more), nutrition status (steady or non-steady) (Walker and Pitman, 1976). The conventional compartmental analysis is suited to determine unidirectional fluxes and compartmental contents of ions in excised root tissues, or suspension-cultured cells (Pitman, 1963; Cram, 1968; Poole, 1971; Macklon, 1975a; Pfrüner and Bentrup, 1978; Jeschke and Jambor, 1981). Since it was considered to be small in excised roots (Macklon, 1975), the xylem transport in intact plants was not included in this conventional model (Pitman, 1963; Etherton,1967; Pallaghy et al., 1970). However, the method was modified by Pitman (1971, 1972) to study Cl^- uptake and transport in barley roots. Tracer efflux from the

cortical cell surface and the transport of tracer into the xylem were measured and analyzed separately. A three compartment model, including xylem transport, was tested in the study of unidirectional fluxes of Na^+ in roots of intact sunflower seedlings (Jeschke and Jambor, 1981). In two compartment models, xylem transport was also considered in studies of $^{13}NO_3^-$ fluxes in roots of intact barley seedlings (Lee and Clarkson, 1986; Siddiqi et al., 1986). The two compartments included the cell wall and cytoplasm, respectively. The short half-life of ^{13}N decay (9.98 min) precluded analysis of the vacuole.

The testing model for higher plants by compartmental analysis (Walker and Pitman, 1976) is based on the assumption that (1) the cytoplasm and vacuole are in series; (2) the cytoplasmic content is very much less than the vacuolar content; (3) the tissue is in a steady state (Cram, 1968). Therefore one may expect that at steady-state conditions of roots:

$$S_c = S_o (1 - e^{-k_c t}) \qquad [3]$$

when roots are exposed to a radioisotope-labeled medium with specific activity S_o, the radioisotope content of the cytoplasm S_c increases exponentially with time (t) and the rate of tracer exchange of the cytoplasm (k_c) is given by the relationship ($k_c=0.693/t_{1/2}$). The quantity of radioactivity inside the cell Q_c^* is given by

$$Q_c^* = A\ t\ \phi_{oc}\ S_c \qquad [4]$$

where A is a cross section constant and ϕ_{oc} is the flux from outside to cytoplasm. The fluxes in opposite directions, between cytoplasm and vacuole are considered to be equal at steady state:

$$\phi_{cv} = \phi_{vc} \quad [5]$$

then the flux into the cytoplasm

$$\phi_{oc} = \phi_{co} + \phi_{cx} - \phi_{xc} \text{ ; (if } \phi_{xc} << 0 \text{ it may be neglected)} \quad [6]$$

therefore net uptake of an ion

$$J_{oc} = \phi_{oc} - \phi_{co} \quad [7]$$

and the transport of ion from root to shoot through xylem would be

$$J_{ox} = \phi_{ox} - \phi_{xc} \quad [8]$$

if roots were uniformly labeled after 16-24 hours loading:

$$S_v = S_c = S_o \quad [9]$$

and the specific activity in the xylem can be estimated from the transport rate of tracer ($\Phi_{cx}(t)$) and transport rate of ion ($J_{ox}(t)$) with the assumption that the symplasm behaves like a rapidly mixed phase and has a uniform specific activity S_c

$$S_x = \Phi_{cx}(t) / J_{ox}(t) \quad [10]$$

Based on these relationships, one is able to estimate unidirectional fluxes and other parameters for each of the compartments.

A biphasic efflux pattern suggests two phases, outside and inside the plasma membrane (Lüttge and Higinbotham, 1979). Since the fastest component was found in both living tissue and chloroform-killed tissue, Cram (1965) concluded that the fastest component of efflux of tracer Cl^- from carrot tissue probably corresponded to the apparent free space (**AFS**). After treating barley roots with either sodium dodecyl sulphate

(SDS) or 70°C hot-water for 30 min, the amounts of released $^{13}NO_3^-$ during initial efflux were similar to the control plants (Siddiqi et al., 1991). Therefore this rapid efflux component probably corresponds to the AFS. Another approach has been to use different sizes of molecules to confirm the AFS phase. It was found that [1,2-^{3}H] polyethylene glycol (^{3}H-PEG) is too large to diffuse into AFS, but D-[1-^{14}C] mannitol is able to diffuse freely in the AFS without been absorbed by root cells (Shone and Flood, 1985). After loading with a mixture of ^{3}H-PEG and D-[1-^{14}C] mannitol, plant roots were washed in unlabeled solution. Since the ratio of ^{3}H and ^{14}C should be same from the surface film of 'loading' solution carried over with the roots, the extra D-[1-^{14}C] mannitol must be washed out from AFS, and can be used to assess the volume of the AFS. It was found that there was an initial rapid release of 90% of ^{3}H and ^{14}C within the first 1 min but more ^{14}C was subsequently released (Lee and Clarkson, 1986). Therefore the rapidly released radioactivity during early efflux is probably from the AFS.

A tricompartmental efflux pattern (including the apparent free space) were reported for Cl^- in carrot root slides or isolated corn root cortex (Cram, 1968; 1973), and excised or intact barley roots (Pitman, 1963, 1971); and for Na^+ and K^+ in intact barley roots (Poole, 1971a, 1971b; Jeschke, 1982). Based on the results of compartmental analysis and other studies, Cram (1965) concluded that, in addition to the fastest efflux from the AFS, the two slower components were considered to be subcellular in origin, the cytoplasm and the vacuole. Further quantitative considerations and model fitting suggested that the cytoplasm and the vacuole are arranged in series with direct connection between the external solution and the cytoplasm, but not between the external solution and the vacuole (MacRobbie, 1964; Cram, 1965).

Also a third small symplastic kinetic compartment may exist in addition to the bulk cytoplasm and vacuole (Lüttge and Higinbotham, 1979; Lazof and Cheeseman, 1986). In a study of sodium transport in *Spergularia marina*, Lazof and Cheeseman (1986) found that the rapid fluxes involved only a very small portion of the total Na^+ in the roots but the authors were unable to identify the physical entity corresponding to the compartment identified. There were also several similar reports in other transport studies. The additional compartment could be the small portion of the bulk cytoplasm connecting to the vacuole (Pitman, 1963); or the cytoplasm can exchange with both vacuole and plastids (Walker and Pitman, 1976); or the possible involvement of vesicles moving in the cytoplasm (Dodd et al., 1960; Lüttge and Osmond, 1970); or the involvement of vesicular transport of ER (Arisz, 1960; MacRobbie, 1970; Stelzer et al., 1975; Tanchak et al., 1984).

2.4.4. The general procedures of compartmental analysis

The general procedure for compartmental analysis has been described in detail (Walker and Pitman, 1976; Zierler, 1981; Rygiewicz et al., 1984). Several radioisotopes have been used in compartmental analyses, $^{36}Cl^-$, $^{82}Br^-$, $^{42}K^+$ or $^{86}Rb^+$, $^{22}Na^+$, $^{45}Ca^{++}$, and $^{28}Mg^{++}$. One part of this technique involves the use of radioisotopic tracers to measure influx and efflux, the separate components of the net flux. The second part is a more systematic method to analyse the kinetics of movement of ions to or from different compartments (Cram, 1968). The basic assumption of this procedure is that radioisotope loaded into different compartments will be washed out with different rate constants.

After allowing plant tissues, cells or roots to load with radioactive tracer for a designated duration, the efflux of this radioisotope is measured for a prescribed period of time. Depending on the type of ion studied, there are two ways to count the radioactivity. For nonmetabolized ions, such as Cl^-, Br^-, K^+, Na^+, Ca^{++}, and Mg^{++}, the radioactivity remaining in the tissue at the end of elution can be counted. By counting the eluates at different times the counts remaining in the tissue at these times can be estimated. For metabolized ions, however, counts remaining in the tissues would be misleading because they consist of the radioactive ion under examination and the metabolic products of its assimilation. In the latter case the rate of efflux, rather than counts remaining must be estimated as a function of the duration of elusion. However, even this method requires that the identity of the effluxed ion be confirmed.

Plotted as a function of time on a semi-logarithmic plot, the activity data (e.g. cpm remaining in system or efflux rate) are resolved into different linear phases which have been interpreted as corresponding to different compartments within the cells. One flaw in this method has been the subjective basis of line fitting (curve-peeling) of data which has implications for the number of exponential terms and their coefficients. To improve the method, Rygiewicz et al. (1984) proposed a microcomputer method in which maximization of r^2 for linear regression serves as the criterion to determine data points belonging to each compartment. This development greatly increased the accuracy of parameter estimation (Rygiewicz et al., 1984) and the objectivity of the estimated results (Cheeseman, 1986).

Selected parameters obtained from compartmental analysis from several sources are shown in **Appendix B.** It was reported that the half-

lives of Cl^- exchange for apparent free space, cytoplasm and vacuole were 1.4 min, 10 min and 300 h, respectively for carrot root tissue (Cram, 1968). In excised barley roots, a slow, vacuolar compartment, was not visible even after 10 h of exchange (Behl and Jeschke, 1982). It must be kept in mind that compartmental analysis alone does not allow one to identify each compartment (Lüttge and Higinbotham, 1979), one must interpret the results with necessary caution and verify these correlations independently. For example, several techniques are available to identify and quantify the vacuole (Clarkson and Lüttge, 1984).

2.4.5. Procedures for compartmental analysis in the present study

For better time control of the separation of 'washing' solutions from the $^{13}NH_4^+$-labeled roots during the efflux process and to reduce disturbance of roots, I devised a simple apparatus in which to perform the efflux study. The spout of a plastic funnel (100 mm diameter) was cut to fit into the barrel of a 25 cc plastic syringe, into which it was sealed. A length of rubber tubing replaced the needle end of the syringe and a metal spring clip on the tubing functioned as drainage control. A small hole was drilled in the wall of the syringe barrel near the bottom, and a needle introduced through this hole to provide for aeration. This technique also resulted in good mixing of the 'washing' solution.

Roots of rice seedlings used for compartmental analysis were immersed for 30 min in the 'loading' solution. These pre-labeled roots were carefully introduced into the syringe barrel for elution. Samples of 20 ml 'washing' solution were poured into the efflux-funnel and allowed to exchange with the ^{13}N-labeled roots. After prescribed intervals, this

solution was drained from the funnel directly into a 20-ml scintillation vial, by opening the drainage clip. Fresh 'washing' solution was poured into the efflux-funnel from the top of the funnel, immediately after closing the drainage clip. The duration of successive washes were: 1 x 5 s, 1 x 10 s, 7 x 15 s, 2 x 30 s, 5 x 1 min and 5 x 2 min. After the last wash, the plants were cut into shoots and roots and introduced into separate scintillation vials. The radioactivities of all samples were counted immediately. In order to be assured that the ^{13}N species that had effluxed from the roots was $^{13}NH_4^+$ rather than any metabolic products, two other sets of $^{13}NH_4^+$-labeled roots were effluxed for 30 min in 750 ml 'washing' solution. Two 20-ml samples of the efflux solution from each beaker were taken and separated by the CEC procedure (see below) and counted for radioactivities. The radioactivities released from intact rice roots into efflux solutions during 18 min efflux experiments, were counted, converted to efflux rates and plotted versus time in semi-log plots (see Fig. 2 in section 3.3.1.). This method of analysis is required because NH_4^+ is rapidly metabolized in rice roots (Yoneyamo and Kumazawa, 1974), and converted into amino acids and proteins. As a consequence, standard methods of compartmental analysis (Walker and Pitman, 1976), based on semi-log plots of cpm remaining in the tissue plotted against time are not appropriate. Hence the values of log of rate $^{13}NH_4^+$ released against time were plotted using the methods detailed by Lee and Clarkson (1986) in an automated computer analysis (Siddiqi et al., 1991).

2.5. Determination Of Ammonium

Intracellular NH_4^+ was extracted from rice roots by use of a Cation Exchange Column (CEC) separation based on the methods of Fentem et al., (1983a) and Belton et al., (1985) and determined by the indophenol blue colorimetric method (Solorzano, 1969). The procedure was as described in Wang et al., (1993a): in brief, after desorbing in NH_4^+-free MJNS for 3 min to remove NH_4^+ in the cell wall, the roots were cut, weighed, and ground with liquid nitrogen in a pre-cooled porcelain mortar and extracted with 10 ml of 10 mM sodium acetate buffer (pH 6.2). The resulting slurry was passed through a Whatman #1 filter paper and then washed 3 times each with 5 ml of the same buffer solution. The filtrate was passed through the CEC filled with 3 ml of resin (Dowex-50, 200-400 mesh, Na^+ form). The NH_4^+ adsorbed on the CEC column was eluted using 250 mM KCl. The concentration of NH_4^+ in solution was determined by the indophenol blue colorimetric method (Solorzano, 1969).

2.6. Preparation Of Metabolic Inhibitors

The same metabolic inhibitors were used in the $^{13}NH_4^+$ influx study (Chapter 5) and electrophysiological study (Chapter 6). The inhibitors used were as follows: (1) CCCP (10 μM): carbonylcyanide m-chloro-phenylhydrazone dissolved in ethanol; (2) CN^- plus SHAM (1 mM): NaCN plus salicylhydroxamic acid dissolved in water. The resulting alkaline pH was adjusted by titration with H_2SO_4 to pH 6; (3) DES (50 μM): diethylstilbestrol dissolved in ethanol; (4) DNP (0.1 mM): 2,4-dinitrophenol dissolved in ethanol; (5) Mersalyl (50 μM): Mersalyl acid dissolved in water; (6) *p*CMBS (1 mM): *p*-chloromercuribenzene-sulfonate dissolved in

water. The acidic pH was adjusted by titration with $Ca(OH)_2$ to pH 5.8. Ethanolic solutions of CCCP, DES and DNP were added to the nutrient solutions to give a final ethanolic concentration of 1%. Control solutions were treated with ethanol at the same concentration.

2.7. ELECTROPHYSIOLOGICAL STUDY

2.7.1. Transmembrane electrical potential measurement

Usually plant cell transmembrane potential differences are in the range of -100 to -200 mV negative inside (Higinbothan, 1973; Tester, 1990). In the early 1930's, Umrath started to use microelectrodes to measure the membrane potential across the tonoplast (Findlay and Hope, 1976). Since then, other electrical properties of plant cells have also been studied such as membrane capacitance (Curtis and Cole, 1938), membrane conductances (Cole and Curtis, 1939), and membrane resistance (Higinbotham et al., 1964; Spanswick, 1970; Anderson et al., 1974). The contemporary climax of electrophysiology occurred when Neher and Sakmann (1976) developed of patch-clamping techniques. The combination of molecular gene cloning and patch-clamp analysis (Hedrich et al., 1987) represents a particularly powerful means of elucidating the mechanism of ion transport through cell membranes.

The chemical potential of an ion (j) is composed of all those components that enable it to do work and can be expressed by the equation [11]:

$$\mu_j = \mu^o_j + RT \ln a_j + z_j F\Psi + V_jP + m_j gh \qquad [11]$$

where μ_j is the chemical potential of the ion j in joules mol^{-1} and μ^o_j is the standard state chemical potential of 1 mole of the ions j per liter at 0°C; R is the gas constant (8.314 J mol^{-1} $°K^{-1}$); T is absolute temperature in °K (°K = 273 + [°C]); a_j is the activity of the ion; z_j is its valency; F is the Faraday constant (9.65×10^{-4} J $mol^{-1}V^{-1}$); Ψ is the electrical potential in volts; V_j is the volume; P is the pressure; m_j is the mass; g is the gravitational acceleration; and h is the height above sea level. In terms of solute transport across the membrane, V_j is very small and Δh is generally negligible. When the concentration (C_j) of the solute is low so that the activity and concentration are close, concentration C_j (mol m^{-3}) can be used in place of the activity a_j ($a_j = \gamma_j C_j$), where γ_j is the activity coefficient.

Simple diffusion is a non-mediated transport process whereby the solute moves along the free energy gradient. In addition to the lipid composition, the difference of ion concentration just inside and outside the plasma membrane determines the diffusion of solute across a membrane. Ion diffusion through membranes may be described by the permeability coefficient which is the flux per unit driving force (in its original conception, the concentration gradient). For the diffusion of small noncharged molecules such as NH_3 and H_2O, the chemical potential

$$\mu_j = \mu^o_j + RT \ln C_j \qquad [12]$$

can be expressed as in equation [12]. Since the driving force is only due to the concentration gradient from high to low (negative sign), the net flux J_j (mol m^{-2} s^{-1}) is expressed as in equation [13]:

$$J_j = K_j C_j (-d\mu_j / dx) \qquad [13]$$

Differentiating in equation [6] and replacing $K_j RT$ (in equation [14]) by D_j (the diffusion coefficient) (Stein,1986) gives equations [14] and [15]:

$$J_j = - K_j \ RT \ d C_j / dx \qquad [14]$$

$$J_j = - D_j \ (d C_j / dx) \qquad [15]$$

Equation [15] is Fick's First Law of diffusion, where K_j is the proportional coefficient or the mobility of the ion *j*, and D_j is the diffusion coefficient of species *j* in m^{-2} s^{-1}. If P_j (m s^{-1}) is the permeability coefficient of the medium or the membrane for ion *j*, then

$$P_j = - D_j / \Delta x \qquad [16]$$

therefore, for the concentration gradient $\Delta C_j = C^o_j - C^i_j$

$$J_j = P_j \ \Delta C_j = P_j \ (C^o_j - C^i_j) \qquad [17]$$

The permeability (P_j) of a chemical species (j) is a measure of the ability of the species of small non-electrolyte to pass through a membrane. The permeability coefficient for isopropanol or phenol is 10^{-6} m sec^{-1} across the plasma membrane (Nobel, 1983).

The diffusion of most ions across the membrane is very low due to their low permeability compared to non-electrolytes. In addition to the concentration gradient, the electrical potential gradient must be included in the driving force. Therefore, equation [11] can be presented as:

$$\mu^*_j = \mu^o_j + RT \ln C_j + z_j F\Psi \qquad [18]$$

For a particular ion, the electrochemical potential gradient (μ^*_j) determines the potential for passive ion flux. At equilibrium both outside and inside electrochemical potentials are the same:

$$\Delta\mu^*_{io} = \mu^*_o - \mu^*_i = 0 \qquad [19]$$

combining equation [18] and [19]

$$\Delta\mu^*_{io} = (RT \ln C_i + zF\Psi_i) - (RT \ln C_o + zF\Psi_o) \qquad [20]$$

where : $\Delta\mu^*_{io}$ is the electrochemical potential difference across the membrane; μ^*_o and μ^*_i are the electrochemical potential outside and inside the cell membrane respectively, Ψ_i and Ψ_o represent the inside and outside electrical potentials, respectively, measured as V; C_i and C_o are the concentration (mM or mol m^{-3}) inside and outside the cell membrane, respectively.

Because of the selective and permeable nature of membranes and the existing concentration asymmetry, the electrical potential difference at zero net flux, when $\Delta\mu_{io} = 0$, is defined as the Nernst potential (Ψ_N) as in equation [21]:

$$\Psi_N = \frac{RT}{zF} \ln \left(\frac{C_o}{C_i}\right) \qquad [21]$$

This is the Nernst equation which describes the electrochemical potential of an ion distributed at thermodynamic equilibrium between two phases separated by a cell membrane. Considering monovalant cations and assuming temperature to be 25°C equation [21] can be simplified to [22]:

$$\Psi_N = -\ 59 \log \left(\frac{C_o}{C_i}\right) \qquad [22]$$

When $\mu^*_{io} \neq 0$, equation [20] and [21] can be rearranged as:

$$\Delta\mu^*_{io} = zF\ ((\Psi_i - \Psi_o) - (RT \ln C_o - RT \ln C_i)/zF)$$

$$= zF\,(\Psi_M - \Psi_N) \qquad [23]$$

where Ψ_M is the measured membrane electrical potential differences across the membrane in volts ($\Psi_M = \Psi_i - \Psi_o$), normally this potential difference across the plasma membrane is large for plant cells (about -200 mV), negative inside (Dainty, 1962; MacRobbie, 1971; Higinbotham, 1973).

The membrane potential (Ψ_M) can be generated from three sources (Nicholls, 1982). One is due to diffusion potentials which may contribute 30 to 40% of measured membrane potential (Pierce and Higinbotham, 1970; Higinbotham et al., 1970). Salts (e.g. KCl and NaCl) in solution dissolve to release cations (K^+ and Na^+) and an anion (Cl^-) which may have different membrane permeability (P_{K^+}, P_{Na^+} and P_{Cl^-}). Presuming that there is initially no electrical asymmetry across the membrane, when ions move along their chemical potential gradient, different mobilities of cations and anions result in charge separation which creates an electrical potential difference, known as a diffusion potential (Ψ_D). It can be assessed by the Goldman voltage equation:

$$\Psi_D = \frac{RT}{F} \ln \left(\frac{P_K[K]_o + P_{Na}[Na]_o + P_{Cl}[Cl]_i + \ldots}{P_K[K]_i + P_{Na}[Na]_i + P_{Cl}[Cl]_o + \ldots} \right) \qquad [24]$$

The second source of membrane potential is the Donnan potential, though the contribution is relatively small. Inside the plant cell, there are many large organic molecules, such as protein and other large polymers (RNA and DNA), with a large number of immobile carboxyl, phosphate and amino groups from which H^+ can dissociate. The asymmetrical distribution of diffusible cations leads to a small negative potential across the plasma membrane (negative inside) (Nobel, 1983).

Thirdly, a major component is a metabolically-driven potential due to the operation of an electrogenic ion pump - the H^+ pump. The H^+ pump (H^+-translocating ATPase) carries a net positive charge across the membrane and contributes directly to the membrane potential (Poole, 1973; Sza, 1984). The activity of H^+ pump depends on the hydrolysis of ATP catalyzed by a plasma membrane ATPase (Hodges, 1973; Poole, 1978; Spanswick, 1981). From equation [20], one can obtain an equation which calculates the electrochemical potential difference for proton at 25°C:

$$\Delta\mu^*_{H^+} = \Delta\Psi + 59\ \Delta pH \qquad [25]$$

A proton concentration difference (ΔpH) and an electrical potential difference ($\Delta\Psi$) are two related entities that make up the electrochemical difference generated in part by the H^+-translocating ATPase (Sze, 1984). By actively pumping out H^+ across the plasma membrane, a 'proton motive force' is built up which can provide the free energy necessary to transport other ions, both actively and passively into the cell (Poole, 1978). In other words, the H^+-pump generates both a potential difference ($\Delta\Psi$) to drive electrogenic uniport, and an electrochemical gradient of protons to drive transport of ions in antiport or symport with H^+.

Since the electrochemical potential difference ($\Delta\mu^*_{io}$) across a membrane is the combined chemical potential and electrical potential difference (equation [18]), it is used to describe the free energy status of a solute in a particular location. It is assumed that a difference of free energy between two points of a system represents the driving force for a passive flux of ions from one point to another. When the resultant chemical potential difference is just balanced by the resultant electrical potential difference ($\Delta\mu^*_{io} = 0$), there is no net flux of solute by passive forces.

Alternatively it can be stated that no energy is expended in moving ions between the two locations.

2.7.2. Single impalement and membrane potential

Microelectrodes are commonly prepared from a micropipette filled with electrolyte solution. It is a filament-containing or single-barreled borosilicate glass capillary tube with the fine-tip which is pulled with either a vertical or horizontal electrode puller (Purves, 1960; Findlay and Hope, 1976). The external diameter of the tip should be 0.5% or less of the diameter of the plant cell which it is to impale (Purves, 1960). For cytoplasmic insertion, a tip diameter of 1 to 2 μm is usually satisfactory (Findlay and Hope, 1976). A tip diameter of <0.5 μm has often been used (Kochian et al., 1989; Ullrich and Novacky, 1990; Glass et al., 1992). However, the smaller the tip diameter the higher the tip potential or electrical resistance (Findlay and Hope, 1976).

Membrane potential difference can be easily expressed in a number of equations (refers to section 2.7.1.), such as the Nernst potential (Eq. [21]), or electrochemical potential (Eq. [24]), or the Goldman diffusion potential (Eq. [21]). When the potential difference is measured by inserted microelectrode, the value is an apparent resting potential which is the real potential difference plus the total offset potential (Purves, 1981). The total offset potential includes the liquid junction potential, the tip potential and the potential due to the possible dissimilarity between the indifferent electrode and the electrode which contacts the microelectrode's filling solution. The latter can be compensated by the offset control of the oscilloscope amplifier. The liquid junction potential occurs between the microelectrode's filling solution and the electrolyte outside the tip. It can

be decreased by use of 3 M KCl as filling solution since the diffusion coefficients of K^+ and of Cl^- are almost identical.

The tip potential is due to the characteristics of glass wall, electrolyte concentration difference between inside and outside of the tip of micropipette and can be eliminated by filling the micropipette with low pH solution or other treatments (Purves, 1960). A tip potential of -5 to -30 mV was recorded for the microelectrode filled with 0.5 M KCl plus 0.1 M Mes (pH 5) (Ullrich and Novacky, 1990). The electrolyte solution could be 3 M KCl at pH 2.0 (Kochian et al., 1989; Glass et al., 1992) to get a high concentration of ions in the tip and a low electrical resistance. As pointed out by Purves (1960) the history of microelectrode technology can be regarded as a succession of attempts to minimize tip diameter and resistance simultaneously.

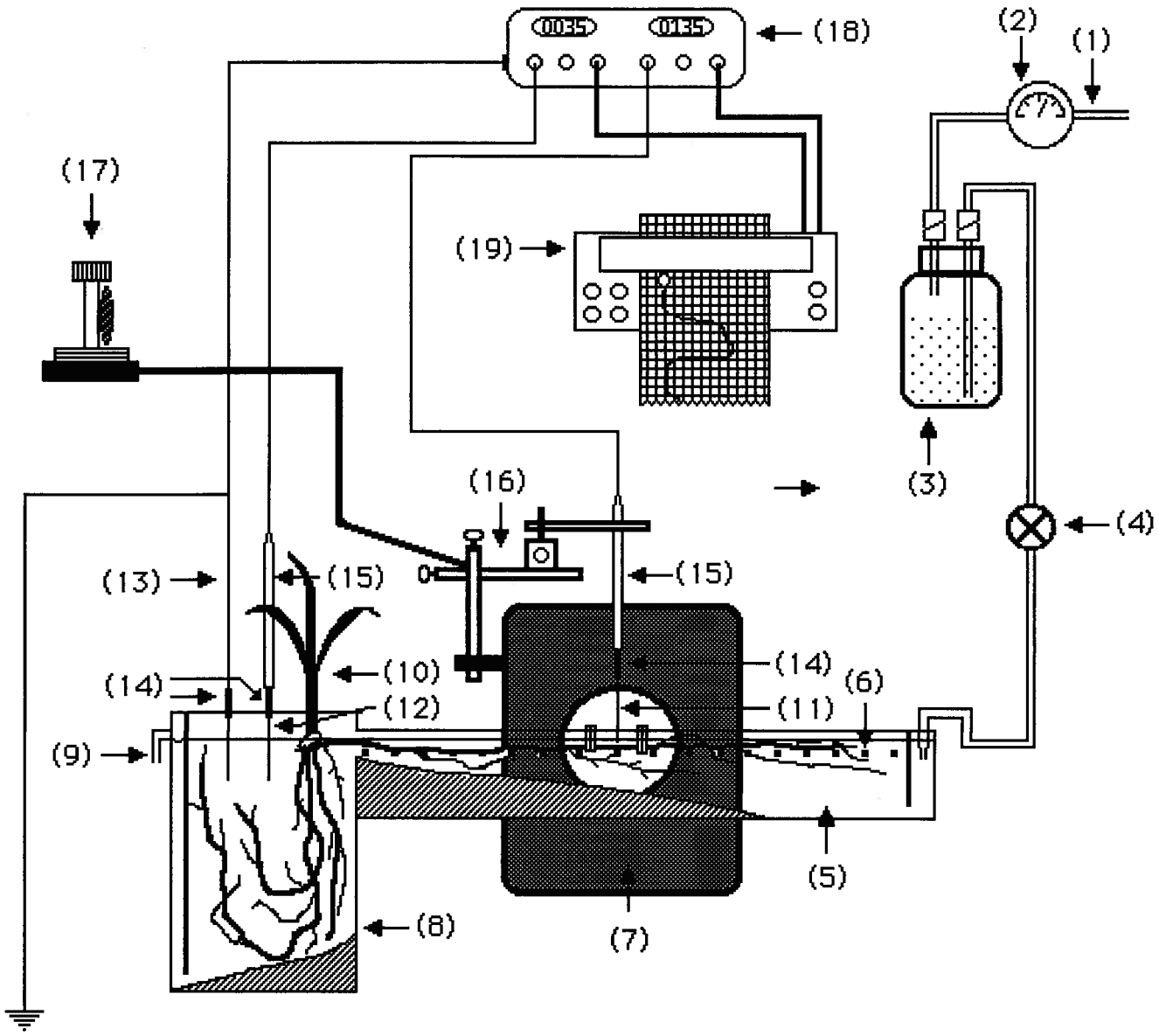
2.7.3. Setup for measuring membrane potential

The fundamental setup for measuring electrical potential difference between two aqueous phases (cell ambient and cytoplasm), is an electrical circuit which should be connected by a salt-bridge, i.e. Hg_2Cl_2 plus KCl (Willians and Wilson, 1981). The microelectrode is such a micro-salt-bridge or miniaturized Calomel half-cell, and connected to the circuit with the silver wire or silver/silver pellet (Purves, 1960). Besides the microelectrode which impales the cell cytoplasm, another reference microelectrode (or the indifferent electrode) is also immersed in bathing solution and connected to the ground. The electrical signals are amplified through a preamplifier (or electrometer), and are sent to the output devices such as the oscilloscope, the tape recorder, the pen recorder the

digital voltmeter or the audio monitor (Findlay and Hope, 1976). Since the plant cells are tiny, vivid and fragile, the impalement the cell through the cell wall and cell membrane is operated by three-way micromanipulators (Kochian et al., 1989; Glass et al., 1992) under the microscope on an anti-vibration table (Purves, 1960). A diagram of such a setup is shown in Figure 2.

2.8. Determination of amino acids in root tissue

Free amino acids in root tissue were determined, after the method reported by Fentem et al., (1983a, 1983b), as follows: weighed root samples were ground with liquid N_2 in a porcelain mortar and extracted with 80% aqueous ethanol. After centrifugation (IEC Clinic Centrifuge), the supernatant was transferred to an evaporating flask. The extraction and centrifugation were repeated 5 times. Pooled extracts were evaporated under vacuum at 35°C on a flash evaporator (Buchler Evapomix). The crude extracts were then re-suspended into 5 ml of distilled deionized water. After mixing 5 ml of chloroform with the crude extract, the supernatant (aqueous phase) was collected into an Eppendorf tube (1.5 ml) for further centrifugation and lyophilization. The extracts were derivatized with phenylisothiocyanate (PTC) automatically on an Amino Acid Analyzer (ABI, Model 402A) equipped to derivatize and hydrolyze applied samples, and then separated by HPLC analysis (Separation system, ABI 130A). The amino acid concentrations were determined by the Amino Acid Analyzer and analyzed by means of an ABI 920A data analysis module. The chemicals used as amino acid standards were from Sigma.



1. Compressed air
2. Air-regulator
3. Bathing solution reservoir
4. Needle valve for controlling flow rate
5. Small chamber for impalement
6. Small pins on the wall to support the root
7. Focusing plate of microscope
8. Large chamber for the rest of root
9. Over-flow of the bathing solution (level)
10. Rice plant
11. Impaling electrode
12. Reference electrode
13. Grounding electrode
14. Electrode holder
15. Preamplifier
16. 3-dimensional manipulator (course)
17. 3-dimensional manipulator (fine)
18. Amplifier
19. Chart recorder

Figure 2. Setup for measuring cell membrane electrical potential.

Chapter 3. FLUXES AND DISTRIBUTION OF $^{13}NH_4^+$ IN CELLS

3.1. INTRODUCTION

The short-lived radioisotope ^{13}N ($t_{1/2}$ = 9.98 min) has been used as a tracer in studies of the fluxes of NO_3^- and NH_4^+ into intact roots of corn and barley plants (McNaughton and Presland, 1983; Glass et al., 1985; Lee and Clarkson, 1986; Hole et al., 1990; Siddiqi et al., 1991). It provides a methodology for the measurement of unidirectional fluxes (influx or efflux) across biological membranes over extremely short times and with great sensitivity (McNaughton and Presland, 1983). Because of its strong γ emission, ^{13}N can be determined rapidly and accurately, with little sample preparation, even in intact plants, by gamma counting techniques (McNaughton and Presland, 1983; Cooper et al., 1985; Meeks, 1992).

The major emphasis in studies of N uptake has been upon NO_3^-, reflecting the widely held perception that NO_3^- is the predominant form of N available to crop species. Relatively less is known about the uptake and subcellular partitioning of NH_4^+ in higher plants. Nevertheless in rice cultivation (Sasakawa and Yamomoto, 1978), in forest ecosystems (Lavoie et al., 1992), in Arctic *tundra* (Chapin et al., 1988) and even in winter varieties of cereals growing in cold soils (Bloom and Chapin, 1981), NH_4^+ may represent the more important form of available nitrogen.

It was demonstrated that net fluxes of NH_4^+ into rice roots gradually acclimated between 0.1 and 1 mM external [NH_4^+] so that net flux at steady-state varied little between plants grown in these concentrations (Wang et al., 1991). Nevertheless, there is a lack of information about

fluxes between subcompartments in relation to acclimation or to the mechanism(s) of NH_4^+ uptake. For example, Presland and McNaughton (1986) failed to observe $^{13}NH_4^+$ efflux from maize roots. By contrast, a sizable net efflux of endogenous $^{14}NH_4^+$ was reported in wheat, oat, and barley upon transfer to $^{15}NH_4^+$ solution, although there was no exact correlation between root ammonium concentration and net $^{14}NH_4^+$ efflux (Morgan and Jackson, 1988a, b).

The internal NH_4^+ concentration of plant roots can readily be assayed, after extraction, by methods based on colorimetry or ion-specific electrodes (Fentem et al., 1983a; Morgan and Jackson, 1988a, 1988b; Roberts and Pang, 1992). However, such analyses fail to provide information on the subcellular distribution of NH_4^+. On the basis of biochemical analysis, it was concluded that more than one intracellular pool of NH_4^+ existed in roots of rice (Yoneyama and Kumazawa, 1974, 1975; Arima and Kumazawa, 1977). Two other methods have been employed to determine subcellular NH_4^+ distribution, namely, efflux analysis (Macklon et al., 1990) and the nuclear magnetic resonance spectroscopy (Lee and Ratcliffe, 1991; Roberts and Pang, 1992). These studies recognized several NH_4^+ fractions of roots, corresponding to those of the superficial, water free space, Donnan free space, the cytoplasm and the vacuole.

In this chapter, the results of compartmental analyses, using $^{13}NH_4^+$ efflux, are used to estimate the half-lives of NH_4^+ exchange and the size of major compartments in root cells, as well as NH_4^+ fluxes between these compartments. Together with data obtained from chemical fractionation, it was possible to develop a detailed analysis of the initial fate of absorbed $^{13}NH_4^+$. In addition, the $t_{1/2}$ values for $^{13}NH_4^+$ exchange provide essential

parameters for the design of appropriate protocols for influx measurement, particularly the duration of $^{13}NH_4^+$ loading and post-wash treatments. To evaluate the methodology of the compartmental analyses, influx and net flux of NH_4^+ were also measured by independent methods.

3.2. MATERIALS AND METHODS

3.2.1. Plant growth and ^{13}N production

Details of seed germination, growth conditions, provision of nutrients and production of $^{13}NH_4^+$ are described in Sections 2.2., 2.3., 2.4., and 2.5., respectively.

3.2.2. Measurement of fluxes

3.2.2.1 $^{13}NH_4^+$ Influx

Checks of the fluxes derived from efflux analysis: After 'loading' for 10, 20, and 30 min, respectively, at steady-state conditions, influx of $^{13}NH_4^+$ was also determined by two independent methods: (1) the accumulation of ^{13}N by seedling roots (see section 2.3.2.); (2) the rate of depletion of $^{13}NH_4^+$ from 'loading' solution.

3.2.2.2. Net NH_4^+ flux

In addition, the net flux of NH_4^+ was also measured based on the rate of depletion of $^{14}NH_4^+$ (see section 2.3.4.).

3.2.2.3. Time course of $^{13}NH_4^+$ uptake

In the time-course experiments, G2 or G100 plants were exposed to 2 μM or 100 μM $^{13}NH_4^+$-labeled loading solutions, respectively, for durations ranging from 10 sec to 31 min. As described in section 2.3.1., roots were subjected to a standard pre-wash, loading and post-wash procedure.

3.2.3. Compartmental Analysis

The procedure for compartmental analysis was followed as described in section 2.4.5.

3.2.4. Partition of absorbed $^{13}NH_4^+$

3.2.4.1. Separation of ^{13}N-compounds in plant tissue

$^{13}NH_4^+$ was separated from its immediate metabolic products by Cation Exchange Column (CEC) Separation described in section 2.5. After plants were loaded in 100 μM $^{13}NH_4^+$ for 30 minutes, the separated, frozen $^{13}NH_4^+$-labeled shoots and roots were first counted in the gamma counter and then ground in liquid nitrogen. After the filtration, the radioactivity remaining on the filter was referred to as root debris. The filtrate was passed through the CEC filled with 3 ml of resin (Dowex-50, 200-400 mesh, Na^+ form) resulting in an elute (Off-CEC) and a CEC-bound fraction (On-CEC). Two sets of G100 plants, containing 100 ~120 plants each, were used.

3.2.4.2 Chemical assay of NH_4^+ in root tissue

Root NH_4^+ contents (Q_i) of G2, G100, and G1000 seedlings were separated and determined as described in section 2.5.

3.2.5. Calculation of flux to vacuole (ϕ_{cv})

The results of CEC separation quantified the un-metabolized $^{13}NH_4^+$ fraction in roots following 30 min $^{13}NH_4^+$ loading. This amount (Q^*_{c+v}) represented the combined values of cytoplasmic (Q^*_c) and vacuolar (Q^*_v) radioactivities that can be converted to a chemical quantity (Q_{c+v}) after dividing by the specific activity of $^{13}NH_4^+$ in the external solution (S_o):

$$Q_{c+v} = Q^*_{c+v} / S_o \qquad [26]$$

The specific activity of $^{13}NH_4^+$ within the cytoplasm (S_c) during loading will increase to its steady-state value according to the rate constant for tracer exchange of the cytoplasm ($k_c = 0.693 / t_{1/2}$) as given in the following equation (Walker and Pitman, 1976).

$$S_c = S_o (1 - e^{-k_c t}) \qquad [3]$$

Thus, if S_o and $t_{1/2}$ are known, S_c can be determined for any particular time (t). By 30 min of loading (equivalent to 4 cytoplasmic half-lives, see Table 2), the specific activity of cytoplasmic $^{13}NH_4^+$ (S_c) is brought to approximately 94% of S_o and $^{13}NH_4^+$ accumulated within the cytoplasm also reaches about 94% of Q_c (in Table 4). Therefore, the proportion of Q_{c+v} transferred to the vacuole is given by:

$$Q_v = Q_{c+v} - 0.94 \bullet Q_c \qquad [27]$$

and from Equation [27], the flux to the vacuole (ϕ_{cv}) can be roughly estimated (Method I). The portion of Q^*_{c+v} that is transferred to the vacuole (Q^*_v) is given by:

$$Q^*_v = (Q_v / Q_{c+v}) \bullet Q^*_{c+v} \qquad [28]$$

The accumulation of tracer in the root vacuole is related to the chemical flux to vacuole (ϕ_{cv}) and the specific activity of the cytoplasm at each interval:

$$Q^*_{v\,(t)} = \phi_{cv} \bullet S_{c\,(t)} \qquad [29]$$

and $$\Sigma\, Q^*_{v\,(t)} = \phi_{cv} * \Sigma\, S_{c\,(t)} \qquad [30]$$

The sum of tracer accumulation within the vacuole Q^*_v ($= \Sigma\, Q^*_{v\,(t)}$) is given by Equation [28], and $\Sigma\, S_{c\,(t)}$ can be calculated for each minute from Equation [3]. Therefore, by means of Method II, it is possible to estimate ϕ_{cv} more rigorously from Equation [30].

3.3. RESULTS

3.3.1. Compartmental analysis

Analysis of the ^{13}N released into 'washing' solutions during compartmental analysis revealed that 99.5% of the radioactivity was retained on the CEC (Table 1). Since positively charged amino acids (arginine, histidine and lysine) represented only 5% of total amino acids in

3-week-old rice roots (Yoneyama and Kumazawa, 1974), I interpreted this result to indicate that $^{13}NH_4^+$ was the predominant N species released from roots and adsorbed on the cation exchange resins.

The influence of $[NH_4^+]_o$ on compartmental analyses was investigated by using G2, G100, or G1000 plants, to represent inadequate, adequate and excess N supply, respectively, prior to efflux measurements. A representative sample of such data (18 min efflux) for G1000 plants is shown in Fig. 3. Three distinct phases, having different slopes with high r^2 values were found for each of the three types of plants tested (G2, G100 and G1000). These compartments were tentatively defined as corresponding to: (I) the superficial solution adhering to roots, (II) the cell wall and (III) the cytoplasm, respectively. The half-lives for exchange ($t_{1/2}$) of these compartments were calculated to be ~ 3 sec, 0.5 to 1 min, and 7 to 8.5 min, respectively (Table 2). According to Duncan's multiple range test, there were no significant differences for these values among plants grown under different concentrations of NH_4^+, except for the cell-wall fraction of G2 plants.

One important part of the compartmental analysis was to calculate the fluxes of NH_4^+ across the plasma membrane of root cells. These calculated fluxes are in good agreement with the values obtained by more direct methods using the same root material (Table 3). Influx (ϕ_{oc}) varied with the NH_4^+ level provided during the growth period. Average NH_4^+ influx values for G2, G100 and G1000 plants were estimated to be 1.32 ± 0.07, 6.08 ± 0.61, and 10.16 ± 0.31 μmol $g^{-1}FW\ h^{-1}$, respectively. Net flux (ϕ_{net}) was estimated by subtracting the estimated values of $^{13}NH_4^+$ efflux (derived from efflux analysis) from the influx of $^{13}NH_4^+$, or by measuring

Table 1. Separation of ^{13}N-labeled compounds by cation exchange column. The loading solution, efflux solution and shoot extract were assayed. Each mean is the average of two replicates ± se.

	Radioactivity adsorbed on CEC
	(% of total cpm in sample)
1) in loading solution	99.7 ± 0.1 (2)
2) in efflux solution	99.5 ± 0.5 (2)
3) in shoot extract	0.7 ± 0.2 (2)

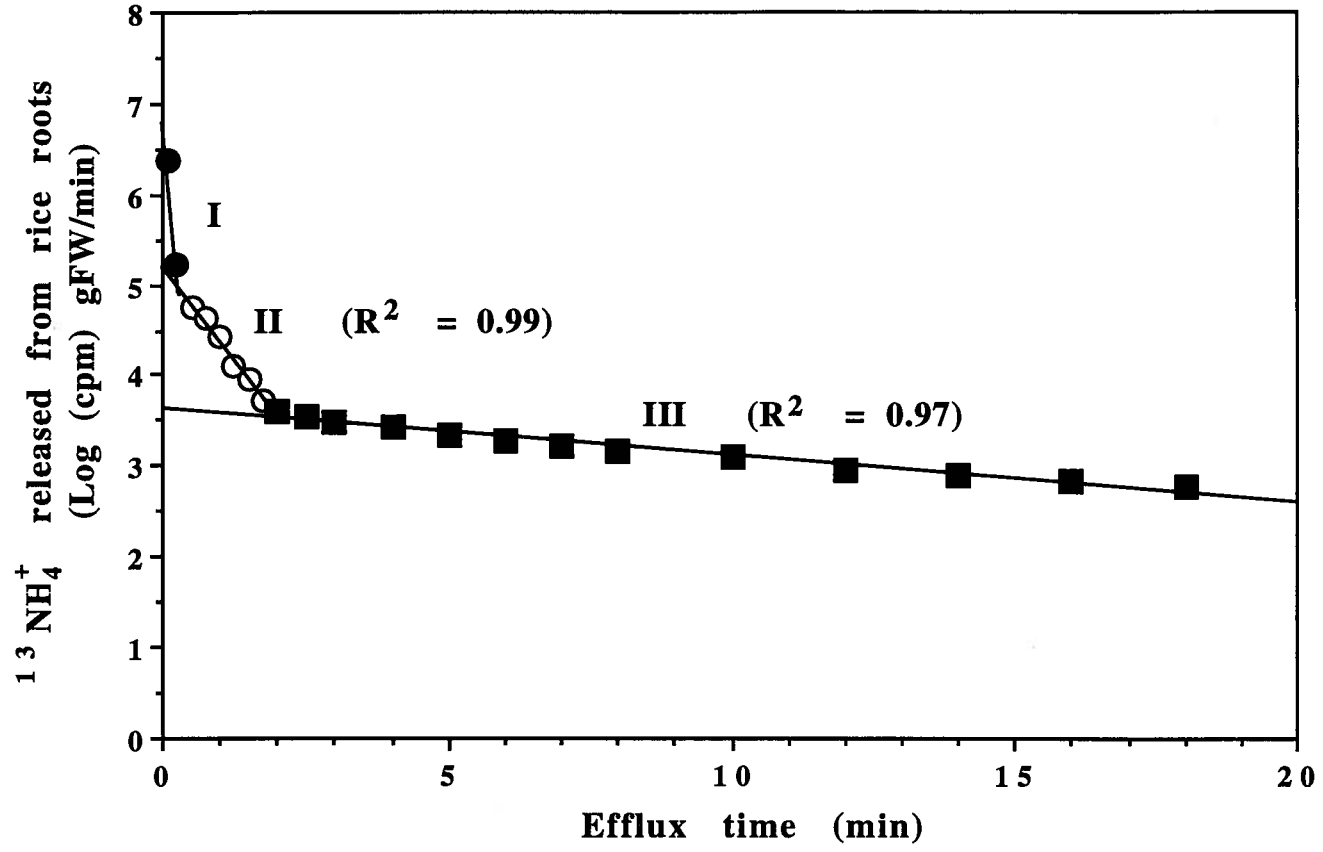


Figure 3. A representative pattern of $^{13}NH_4^+$ released from intact roots. The rate of $^{13}NH_4^+$ (log(cpm) $g^{-1}FW\ min^{-1}$) released from intact rice roots of G1000 plants during 18 min efflux (see text for details). Three phases (I, II, and III) of $^{13}NH_4^+$ releasing were determined by correlation coefficient.

Table 2. Estimated half-lives of $^{13}NH_4^+$ exchange for three compartments of root cells. Means for half-lives of $^{13}NH_4^+$ exchange ($t_{1/2}$) for three compartments (superficial, cell wall, and cytoplasm) were estimated from the efflux analysis. G2, G100 and G1000 plants were loaded in ^{13}N-labeled MJNS for 30 min and effluxed in un-labeled identical MJNS for 18 min at steady-state conditions with regards to $[NH_4^+]_o$. Each mean is the average of 4 individual efflux tests ± se.

	Compartments	G2	G100	G1000
I.	Superficial (s)[a]	3.42 ± 1.00 a	3.83 ± 0.24 a	3.38 ± 0.37 a
II.	Cell wall (min)	1.06 ± 0.10 b	0.57 ± 0.09 a	0.43 ± 0.06 a
III.	Cytoplasm (min)	6.95 ± 1.14 a	7.36 ± 0.12 a	8.33 ± 0.60 a

[a] Duncan's multiple range test was used to compare the means of each compartment. Only means followed by a different letter are significantly different at the 5% level of significance.

Table 3. Comparison of $^{13}NH_4^+$ fluxes across the plasma membrane of root cells. Each mean $^{13}NH_4^+$ fluxes (influx, efflux, and net flux) is the average of 3 or 4 replicates with ± se.

Methods	G2	G100	G1000
Influx (ϕ_{oc}):		(μmol g^{-1}FW h^{-1})	
(1) $^{13}NH_4^+$ efflux analysis [a]	1.20 ± 0.07	5.97 ± 0.41	10.51 ± 2.04
(2) $^{13}NH_4^+$ accumulated in roots [b]	1.39 ± 0.02	5.27 ± 0.20	10.16 ± 0.23
(3) $^{13}NH_4^+$ depletion of medium [b]	1.37 ± 0.02	6.99 ± 0.51	10.29 ± 0.29
(4) $^{13}NH_4^+$ depletion of medium [b]	1.33 ± 0.01	6.11 ± 0.32	9.66 ± 0.63
Net flux (ϕ_{net}):			
(5) $^{13}NH_4^+$ efflux analysis [a]	1.06 ± 0.07	4.80 ± 0.39	7.41 ± 1.55
(6) $^{14}NH_4^+$ depletion of medium [a]	1.11 ± 0.04	4.32 ± 0.15	6.08 ± 0.27
Efflux (ϕ_{co}):			
(7) $^{13}NH_4^+$ efflux analysis[a]	0.13 ± 0.02	1.17 ± 0.14	3.09 ± 0.56
(8) Subtracted (6) from (2)	0.27 ± 0.02	0.94 ± 0.05	4.09 ± 0.04
(9) Subtracted (6) from (4)	0.22 ± 0.17	1.79 ± 0.47	3.58 ± 0.36

[a] Based on 30-min uptake; [b] Based on 10-min uptake.

net depletion of $^{14}NH_4^+$ from the uptake solution. Both methods gave similar results with average values of 1.09 ± 0.03, 4.56 ± 0.24, and 6.75 ± 0.67 μmol NH_4^+ g^{-1}FW h^{-1} for G2, G100 and G1000 plants, respectively. The influx and net flux values of G100 plants were 4 fold higher than those of G2 plants (Table 3). Fluxes of G1000 plants were about 1.5 times the values of G100 plants. Efflux values, expressed as percentages of influx, were 11%, 20%, and 29% for G2, G100 and G1000 plants, respectively, (Fig. 4).

Since the volumes of subcellular compartments are very different (Steer, 1981; Patel, 1990), it is necessary to distinguish between NH_4^+ content (Q) expressed as moles per unit weight of roots (μmol g^{-1}), and NH_4^+ concentration ($[NH_4^+]$) expressed as moles per unit volume of a compartment (μM or mM). The results of estimated cytoplasmic NH_4^+ concentration ($[NH_4^+]_c$), chemically assayed total root NH_4^+ contents (Q_i) of G2, G100 and G1000 plants, as well as calculated values of $[NH_4^+]_i$, $[NH_4^+]_v$, Q_c and Q_v are presented together in Table 4. Values of $[NH_4^+]_i$ and $[NH_4^+]_c$ were higher with higher levels of NH_4^+ provision. The values of $[NH_4^+]_c$, were ~5 to 6 fold higher in G100, and ~10 fold higher in G1000 plants than in G2 plants. The values for the vacuolar pool were based on the differences between the total NH_4^+ content in the roots (Q_i) and the cytoplasmic pool (Q_c). Of the total NH_4^+ of the roots, 92% was localized within the vacuole in G2 plants and about 72% to 76 % in G100 and G1000 plants. Chemical and radioisotopic quantities for various compartments used in calculating ϕ_{cv} are presented in Table 5. The specific activity of cytoplasm ($S_{c\,(t)}$) was calculated for each minute from t=1 to 30 min. Both $\Sigma\, Q^*_{v\,(t)}$ and $\Sigma\, S_{c\,(t)}$ were used for estimating ϕ_{cv}. The ϕ_{cv} estimated by methods I and II are given in Table 5.

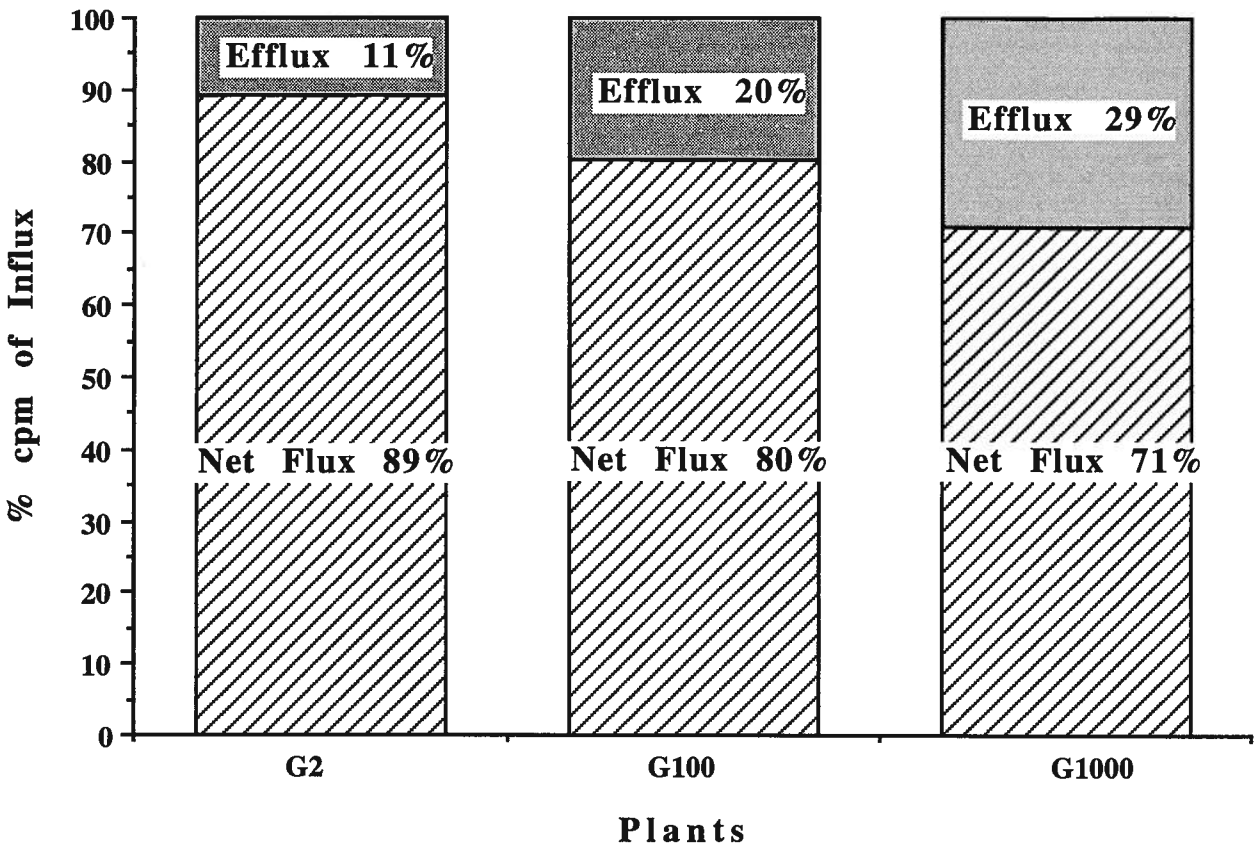


Figure 4. Fluxes of G2, G100, G1000 plants. Efflux (ϕ_{co}) and net flux (ϕ_{net}) as percentage of influx (ϕ_{oc}) for G2, G100 and G1000 plants at steady-state based on the data of compartmental analysis in Table 2.

Table 4. Size of ammonium pools in root cells. Ammonium pools in root cells of G2, G100 and G1000 plants at steady-state. The contents of un-metabolized NH_4^+ in root tissues (Q_i) and cytoplasm (Q_c) and vacuole (Q_v) and their corresponding NH_4^+ concentrations ($[NH_4^+]_c$, and $[NH_4^+]_v$), as well as that of the cell wall pool ($[NH_4^+]_w$), are presented.

Plant	NH_4^+ content			NH_4^+ concentration[a]		
	Q_i[b]	Q_c[c]	Q_v[d]	$[NH_4^+]_w$	$[NH_4^+]_c$	$[NH_4^+]_v$
	(μmol g^{-1}FW root)				(mM)	
G2	2.38	0.19 (8%)	2.19 (92%)	0.56	3.72	2.58
G100	4.31	1.03 (24%)	3.28 (76%)	2.27	20.55	3.86
G1000	6.85	1.94 (28%)	4.91 (72%)	14.41	38.08	5.78

[a] The values of $[NH_4^+]_w$ and $[NH_4^+]_c$ were estimated from compartment analysis with four replicates each and $[NH_4^+]_v$ was estimated from Q_v.

[b] The values of Q_i were obtained from chemical NH_4^+ assay with three replicates each and are the same as the values of $[NH_4^+]_i$.

[c] The values of Q_c were calculated from $[NH_4^+]_c$ based on the assumption that the cytoplasm only had 5% of total cell volume.

[d] The values for Q_v are based on the difference between Q_i and Q_c and the assumption that the vacuole occupies 85% of cell volume. In parenthesis, Q_c or Q_v, respectively, are presented as percentages of Q_i.

3.3.2. Metabolism and translocation of ^{13}N

Virtually none of the $^{13}NH_4^+$ absorbed by rice roots was translocated to the shoots (Table 1). It is improper to express the translocation of ^{13}N (to the shoot) as μmol NH_4^+ per gram fresh weight of roots because (a) ^{13}N is transported from the root in the form of amino acids and (b) the specific activities of these amino acid pools were unknown. Therefore the translocation was expressed as a percentage of the total radioactivity (cpm accumulated in roots plus shoots during the loading period). This total radioactivity is equivalent to net absorption of $^{13}NH_4^+$. Further fractionation of root tissues of G100 plants by the CEC separation revealed that about 8.6% of the radioactivity provided by influx during 30 min $^{13}NH_4^+$ loading was retained in a metabolized form (Table 6). By combining the ^{13}N translocated to shoots (10%) with 'root debris' (4%) and the 'Off CEC' fraction (5%), an estimation of the proportion (19%) of absorbed $^{13}NH_4^+$ that was metabolized during the 30 min was obtained. The partitioning of radioactivity was also calculated based on the total cpm remaining in roots (Table 6).

3.3.3. Time course of $^{13}NH_4^+$ influx in rice roots

The results of steady-state $^{13}NH_4^+$ uptake by G2 and G100 plants, establishing the pattern of $^{13}NH_4^+$ accumulation in rice roots, are shown in Fig. 5. The accumulation of $^{13}NH_4^+$ appeared to be linear for the duration of the 30 min uptake experiments; the coefficient of determination of these lines (0.87 and 0.99 for G2 and G100 plants, respectively) were high. In all cases, the intercept on the ordinate differed significantly from zero (at 5% significance level). G100 plants had a higher accumulation rate than G2

Table 5. Calculation of the flux (ϕ_{cv}) from cytoplasm into vacuole. The data used in calculation were taken from the results of the compartmental analysis (Table 4) and root partitioning experiment (Table 6). The calculation procedure is in section 3.2.4.2.

Parameter		value	unit
S_o		164214	cpm μmol^{-1}
$\Sigma S_{c\,(t)}$	(t=30 min)	3361875	cpm μmol^{-1}
$\Sigma Q^*_{v\,(t)}$	(t=30 min)	79666	cpm g^{-1}
Q^*_{c+v}		238982	cpm g^{-1}
Q_{c+v}		1.46	$\mu mol\ g^{-1}$
Q_c		0.97	$\mu mol\ g^{-1}$
Q_v		0.49	$\mu mol\ g^{-1}$
ϕ_{cv}	(Method I)	0.97	$\mu mol\ g^{-1}\ h^{-1}$
ϕ_{cv}	(Method II)	1.42	$\mu mol\ g^{-1}\ h^{-1}$

Table 6. Distribution of newly absorbed ^{13}N in shoot and root tissues. After 30 minutes loading in ^{13}N-labeled MJNS containing 100 μM NH_4^+, Fractionation of radioactivity in shoots and roots of G100 plants were carried out according to sections 2.5. and 3.2.4.1. Radioactivities are expressed as percentages of total cpm in plants. Each analysis used 100 to 120 plants and data given are means of two replicates (±se).

		% cpm in plant
[A]	in shoots	9.7 ± 0.9
[B]	in roots	
i.	total	90.3 ± 0.9
ii.	% recovery after CEC	
(a)	On-CEC	81.7 ± 2.5
(b)	Root debris	5.1 ± 1.0
(c)	Off-CEC	3.5 ± 0.6
[C]	Metabolized	18.3 ± 2.5

[a] 'Metabolized' is the sum of lines [A] , (b) and (c) based on the total cpm in whole plants or the sum of lines (b) and (c) based on the total cpm in root.

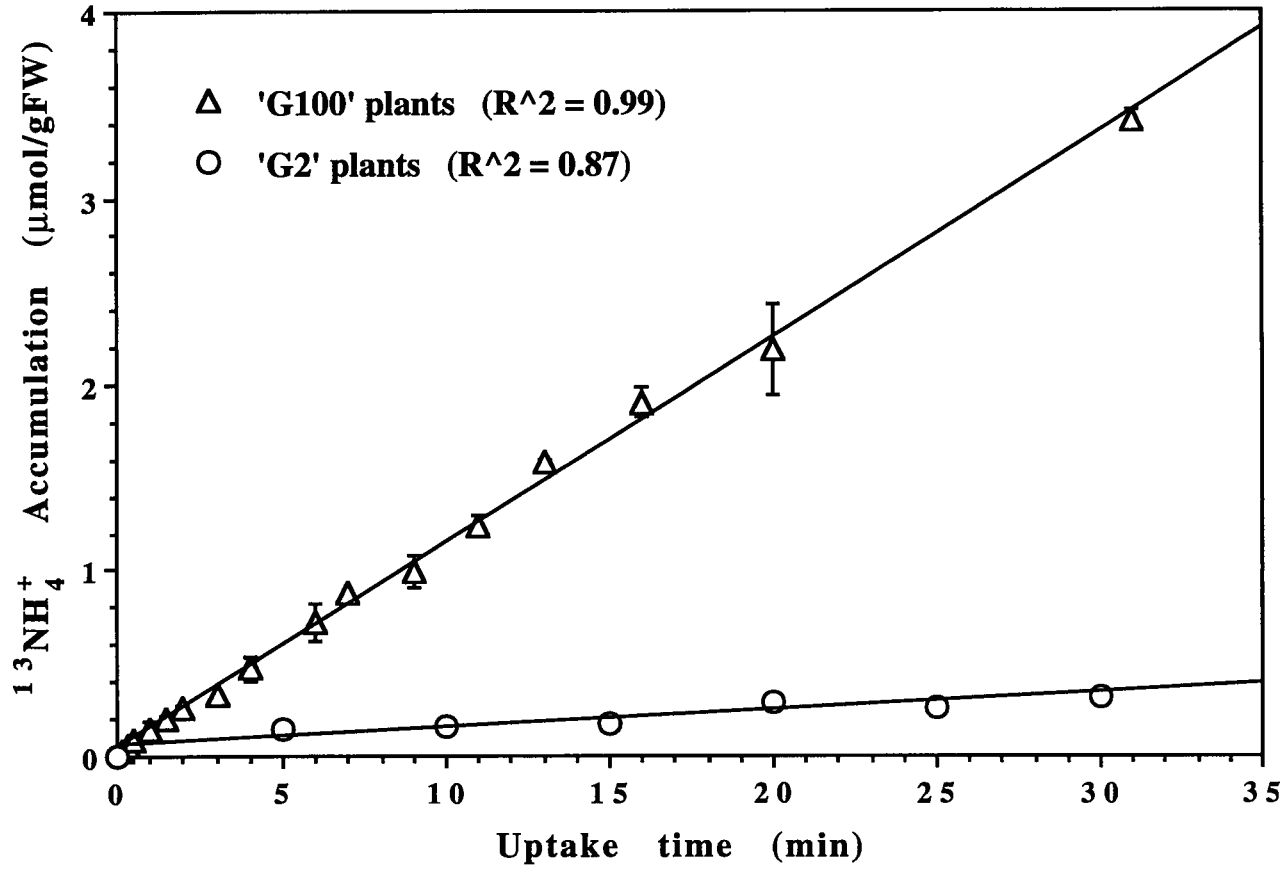


Figure 5. Cumulative uptake of $^{13}NH_4^+$ by G2 and G100 roots. Time course study of $^{13}NH_4^+$ uptake by G2 and G100 roots at steady-state. G2 or G100 rice plants were grown and loaded in ^{13}N-labeled MJNS containing 2 µM (•) or 100 µM (♦) $[NH_4^+]_o$, respectively. Uptake is expressed as the accumulation of $^{13}NH_4^+$ (µmol $g^{-1}FW$). Each datum point is the average of 3 replicates with standard errors as vertical bars.

plants. The data for ^{13}N accumulation were used to calculate the rate of ^{13}N accumulation (influx) as a function of time (Fig. 6). Based upon very short exposures (less than 2.5 min) to $^{13}NH_4^+$, the influx of G100 plants appeared to be about 20 to 30% higher than the steady value of influx. Beyond 5 to 10 min, influx in both G2 and G100 plants remained essentially unchanged; ~1 and 7.5 μmol $g^{-1}FW\ h^{-1}$ for G2 and G100 plants, respectively.

3.4. DISCUSSION

3.4.1. The half-lives of $^{13}NH_4^+$ exchange

Three kinetically distinct phases (I, II, III) with half-lives for $^{13}NH_4^+$ exchange of approximately 3 sec, 1 min and 8 min, respectively, were identified by means of compartmental analysis (Table 2). Phase I is probably due to the surface solution on roots carried-over from the 'loading' solution (Fig. 3). The second phase is attributed to the cell wall fraction, or the apparent free space (AFS) which is the sum of the Water Free Space (WFS) and the Donnan Free Space (DFS) (McNaughton and Presland, 1983 and references therein). The half-life of this phase (0.5 to 1 min) was shorter than the equivalent phase reported for corn roots (2.5 min) by Presland and McNaughton (1986), but similar to the half-life for NO_3^- exchange (0.5 min) in barley roots (Siddiqi et al., 1991). By using the 'efflux-funnel', shorter efflux intervals were achieved. This allowed for resolution of these two rapid phases (I and II) and more accurate estimation of the cell wall half-life.

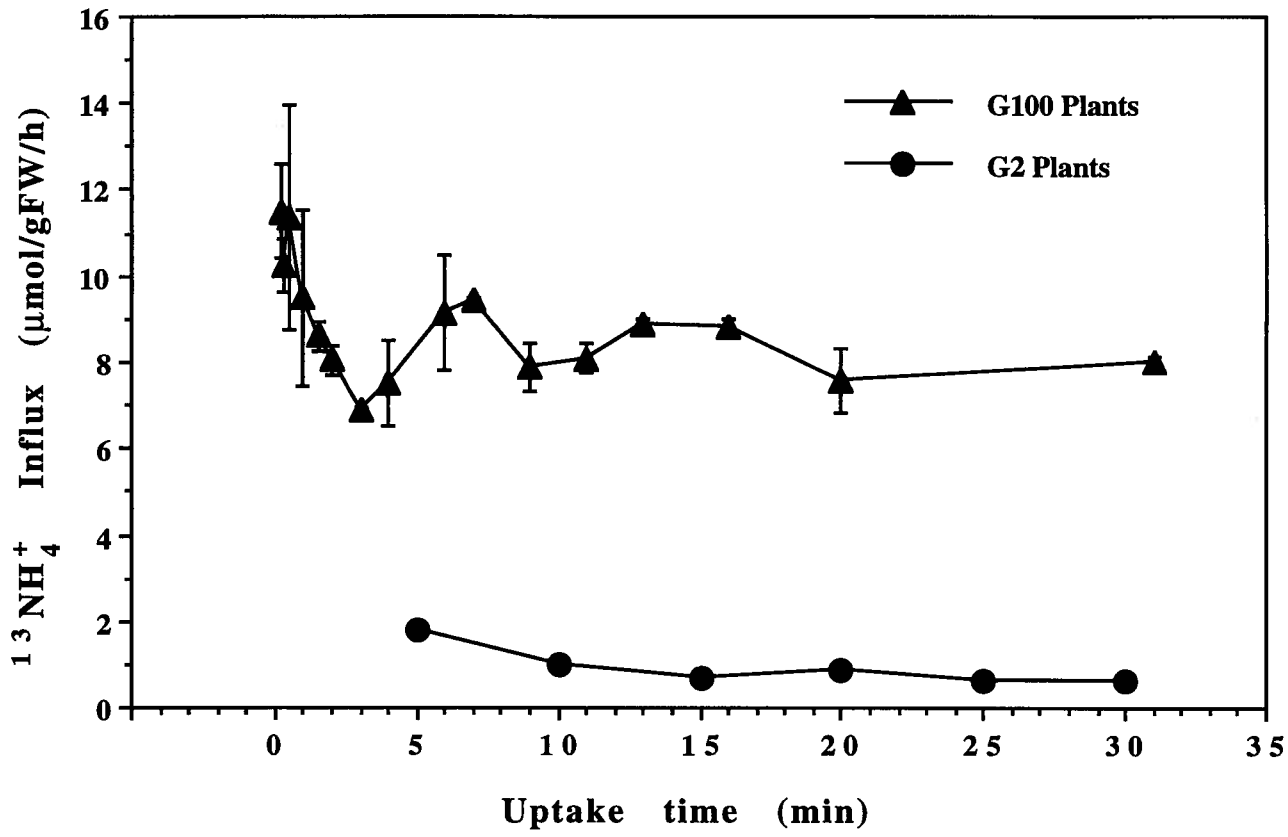


Figure 6. Influxes of $^{13}NH_4^+$ into G2 and G100. Steady-state influxes of $^{13}NH_4^+$ into G2 and G100 roots were measured in the time course study. Symbols are the same as in Fig. 4. Influx is expressed as (µmol g^{-1}FW h^{-1}). Each datum point is the average of 3 replicates with standard error (± se) as vertical bars.

The third phase is believed to be the cytoplasm. The half-lives of cytoplasmic exchange for G2, G100 and G1000 plants ranged from 6.9 to 8.3 min, but the differences were statistically insignificant, although the cytoplasmic pool sizes varied according to the provision of NH_4^+ during growth (Table 2). Siddiqi et al., (1991) showed that barley roots, treated with SDS or pretreated by immersion in water at 70°C for 30 min, accumulated and released significantly less $^{13}NO_3^-$ from phase III, but phase II appeared unaffected. These results were consistent with phase III being the cytoplasm. In studies of $^{13}NH_4^+$ efflux from spruce roots, Kronzucker, H. (personal communication) has found that elevated $[Ca^{2+}]_o$ in the loading and washing solutions reduced the extent of phase II for $^{13}NH_4^+$ exchange in spruce roots, (which had similar half-lives to those observed in rice) as would be expected if this phase corresponded to the cell wall compartment. The short half-life of ^{13}N decay, and long half-life of exchange of the vacuole (Lee and Clarkson, 1986; Macklon et al., 1990), precludes the estimation of vacuolar parameters by efflux analysis using ^{13}N. Using $^{15}NH_4^+$, Macklon et al., (1990) estimated the half-lives for cytoplasmic and vacuolar exchange to be 44 min and 8.2 to 22.8 hours, respectively for excised onion roots. Cooper and Ford (cited in Macklon et al., 1990) observed much shorter $t_{1/2}$ values for cytoplasmic $^{13}NH_4^+$ exchange, ranging from 4 to 10 min in roots of wheat. The latter values are much closer to those obtained in the present studies, i.e. 6.9 to 8 min (Table 2). The longer $t_{1/2}$ values reported by Macklon et al., (1990) may have arisen from species differences and/or differences of methodology.

In order to select appropriate durations for the loading and washing periods employed in influx studies, it is important to estimate the half-lives for $^{13}NH_4^+$ exchange between different compartments (Cram, 1968).

The choice of a 10 min loading time, used in the present study and in subsequent $^{13}NH_4^+$ influx studies, was arrived at from considering the following: (1) the half-life of ^{13}N decay is short ($t_{1/2}$ = 9.98 min) and therefore the influx period should be as short as possible. As the isotope decays, the statistical uncertainty in the measurement of ^{13}N retained by the plant roots or transported to the stem becomes as high as ±15% after about 40 min (McNaughton and Presland, 1983); (2) if the loading time is long, compared to the $t_{1/2}$ for cytoplasmic exchange for $^{13}NH_4^+$, the specific activity of the cytoplasmic pool may approach saturation and the $^{13}NH_4^+$ efflux term (ϕ_{co}) will be maximized. The measured $^{13}NH_4^+$ influx under these conditions would approximate the net flux ($\phi_{net} = \phi_{oc} - \phi_{co}$); (3) although the over-estimation of influx (see below) was minimized by 20 minutes, 10 minutes loading reduced that over-estimation to less then 10% (Fig. 6). The duration of the loading period and the post-wash period is a compromise (Lee and Clarkson, 1986). Since the goal was to measure the unidirectional flux across the plasma membrane (ϕ_{oc}), ^{13}N present in the cell wall should be removed during the post-wash period. Based on the estimated $t_{1/2}$ of the cell wall fraction, a short post-wash period of 3 min (corresponding to 3 to 6 half-lives, Table 1) was adopted in all influx experiments. In order to equilibrate the cell wall fraction to any changes of $[NH_4^+]_o$, rice roots were, therefore, always pretreated for 5 min in identical un-labeled MJNS before loading in ^{13}N-labeled MJNS.

3.4.2. Fluxes of $^{13}NH_4^+$ into root cells

The results of the present study showed that $^{13}NH_4^+$ appeared to be accumulated at a constant rate (r^2 = 0.874 and 0.997, respectively) during

30 min loading of G2 and G100 plants under steady-state conditions (Fig. 5). Moreover, $^{13}NH_4^+$ accumulation increased with increasing $[NH_4^+]_o$ of the loading solution. This observation is similar to previous reports indicating that the accumulation of ^{13}N (either as $^{13}NO_3^-$ or $^{13}NH_4^+$) by plant roots increases in linear fashion during short (usually <15 min) loading periods (Presland and McNaughton, 1984; Lee and Drew, 1986). The data for $^{13}NH_4^+$ accumulation by G2 and G100 plants are also presented as plots of influx versus time (Fig. 6). Influx values based upon very short exposures to $^{13}NH_4^+$ were accompanied by large errors probably associated with the lower counts accumulated and a large multiplicative factor involved in calculating influx on a per hour basis. Nevertheless, the data indicated that initial influx values were 20 to 30% higher than those recorded after 2 to 5 min. After loading for more than 5 min, the influxes were ~1 and 7.5 μmol $g^{-1}FW\ h^{-1}$ for G2 and G100 plants respectively, and notwithstanding some variation, remained reasonably constant for the next 25 min. Presland and McNaughton (1984) noted a higher rate of $^{13}NH_4^+$ accumulation in maize roots during the first 2 min that they attributed to apoplasmic filling. In the present study, although the roots were subjected to a 3 min post-wash, any tracer uptake by rice roots during the post-wash period would represent an over-estimate. The impact of these additional counts would be to over-estimate the calculated influx values at shorter loading intervals due to the multiplicative effect in calculating fluxes on a per hour basis. This effect, which decreases as the duration of the influx period increased, was minimized at about 20 min (Fig. 6). This interpretation is in contrast to that of Lee and Ayling (1993) who argue that the lower counts recorded after 2 to 5 min represent an under-estimate of influx due to release of absorbed ^{13}N or ^{15}N as cytoplasmic specific activity reaches steady-state. I question this interpretation because: (1) the $t_{1/2}$ for

exchange of $^{13}NH_4^+$ from the cytoplasmic phase was ~ 8 min for rice roots grown at various nitrogen conditions (Table 2); (2) the absolute value of the efflux from cytoplasm to outside (ϕ_{co}) varied from 10 to 30% of influx (ϕ_{oc}) according to compartmental of analyses (Table 3). Therefore I consider it unlikely that a significant reduction of measured influx would result from efflux of tracer during the short duration of these exposures.

Values for influx (ϕ_{oc}), efflux (ϕ_{co}) and net flux (ϕ_{net}) of $^{13}NH_4^+$ determined by efflux analyses corresponded very well with those obtained by other (more direct) methods (Table 3). This close correspondence allows us to accept the parameters derived from $^{13}NH_4^+$ compartmental analysis with some degree of confidence. Influxes of $^{13}NH_4^+$ into rice roots under steady-state conditions increased according to the levels of $[NH_4^+]_o$ in the growth media (Table 3). A similar trend was shown for net fluxes determined either by efflux analysis or by depletion methods. Net uptake (ϕ_{net}) tended to show only a small increase as $[NH_4^+]_o$ increased from 100 to 1000 μM (Table 3). This confirms my previous report that net uptake of NH_4^+ was acclimated to $[NH_4^+]_o$ in growth media, although the acclimation was not achieved by G2 plants (Wang et al., 1991). These results demonstrated that NH_4^+ fluxes are closely related to the nitrogen status of plants, which is determined by plant growth conditions.

Estimated effluxes of NH_4^+ from rice roots were about 10, 20 and 29% of the influx values for G2, G100 and G1000 plants, respectively (Table 3 and Fig. 4). In addition, efflux was positively correlated with the $[NH_4^+]_c$ (Table 3 and 4). This result agrees with the suggestion that continuous NH_4^+ efflux may be a common feature of net NH_4^+ uptake by roots of higher plants (Morgan and Jackson, 1988a). Nitrogen efflux (either NH_4^+ or NO_3^-) has been reported to be quite significant, particularly at

elevated concentrations of N (Morgan et al., 1973; Breteler and Nissen, 1982). Indeed, Deane-Drummond and Glass (1983a, b) suggested that nitrate efflux might regulate net uptake by means of a type of 'pump and leak' mechanism. By contrast, Lee and colleagues have emphasized the importance of influx in the regulation of net uptake of nitrate, although nitrate efflux was equivalent to almost 40% of nitrate influx in barley roots (Lee and Clarkson, 1986; Lee and Drew, 1986). Morgan and Jackson (1988a, b) also found a sizable net efflux of endogenous $^{14}NH_4^+$ occurred upon transfer to $^{15}NH_4^+$ solutions in wheat, oat, and barley adequately supplied with nitrate. However an exact parallel between root ammonium concentrations and net $^{14}NH_4^+$ efflux was not observed. Although plasma membrane influx determines the maximum rate of net uptake (Lee and Clarkson, 1986), efflux certainly makes a significant contribution to determining net uptake.

Because of its short half-life, ^{13}N is unsuitable for the determination of vacuolar parameters by efflux analysis. Nevertheless, the combination of $^{13}NH_4^+$ efflux analysis and the CEC separation of ^{13}N products enabled us to estimate ϕ_{cv} using two methods. Both results give values for ϕ_{cv} in the range from 1 to 1.5 μmol $g^{-1}FW\ h^{-1}$. Method (I) is based on the estimated $^{13}NH_4^+$ accumulation during 30 min loading, while method (II) involved the use of S_c values estimated minute by minute from a knowledge of the half-life of cytoplasmic exchange (see section 3.2.5.). Therefore method (II) is probably more refined than the value derived from method I. These values are somewhat lower than those obtained by efflux analysis in onion (Macklon et al., 1990), however the Macklon's study was undertaken at 2 mM $[NH_4^+]_o$, compared to my analyses undertaken with G100 plants at 100

μM NH_4^+. The differences may also reflect the methodology and plants species employed.

3.4.3. The NH_4^+ pools in roots

In the present study, the values of Q_i were in the range from 2.38 to 6.85 μmol $g^{-1}FW$ for roots grown with different levels of NH_4^+ (Table 4). Fentem et al., (1983b) reported a value of 3.2 μmol $g^{-1}FW$ in 9-d-old barley roots grown in 1 mM NH_4^+. For barley, wheat and oat grown in NO_3^- or N-free conditions, the value of $[NH_4^+]_i$ was in the range of 0.4 to 2 μmol $g^{-1}FW$ (Morgan and Jackson, 1988a,b, 1989). However, when plants grown in NH_4^+ or in NO_3^- were pretreated with 0.5-1.5 mM $[NH_4^+]_o$ for various periods of time, the values of Q_i were high and varied from 6 to 35 μmol $g^{-1}FW$ (Lee and Ratcliffe, 1991; Morgan and Jackson, 1988a). The relatively low intracellular NH_4^+ content, particularly, under steady state conditions, may reflect the efficiency of NH_4^+ assimilation (Goyal and Huffaker, 1984).

Irrespective of the $[NH_4^+]_o$ provided during the growth period, the bulk of absorbed NH_4^+ was localized in the vacuole (Table 4). Nevertheless, because of the large size of the vacuole, the values of $[NH_4^+]_v$ were significantly lower than those of the $[NH_4^+]_c$ (Table 4). Increasing $[NH_4^+]_o$ from 2 to 1000 μM, caused $[NH_4^+]_c$ to increase more than 10 fold, while $[NH_4^+]_v$ increased by only ~ 2 fold. Cytoplasmic NH_4^+ concentrations of rice roots estimated in the present study (Table 4) were in the range of reported values for wheat, maize, barley and onion (Fentem et al., 1983b; Cooper and Clarkson, 1989; Macklon et al., 1990; Lee and Ratcliffe, 1991). On the basis of NMR studies of NH_4^+ distribution in root tip of maize, cytoplasmic $[NH_4^+]$ ranging from 3 to 438 μM were reported (Roberts and

Pang, 1992). However, in that study, lower values might be expected since root tips were excised from 2-day-old maize seedlings and maintained without an exogenous source of NH_4^+ during estimation of $[NH_4^+]_c$ by NMR. My indirect estimation of $[NH_4^+]_v$ provided a range from 2.6 to 5.8 mM for G2, G100 and G1000 plants (Table 4). Using ^{15}N, Macklon et al. (1990) reported a similar range (3.9 to 10.9 mM) for $[NH_4^+]_v$ in cortical cells of onion roots. Slightly higher values (15 to 36 mM) for $[NH_4^+]_v$ were estimated in maize roots by ^{14}N-NMR spectroscopy (Lee and Ratcliffe, 1991).

3.4.4. Model of $^{13}NH_4^+$ uptake by rice plants

Despite the widespread use of compartmental analysis to investigate compartmentation of non-metabolized ions, e.g. Cl^- (Cram, 1968), Na^+ (Jeschke and Jambor, 1981), and K^+ (Memon et al., 1985), relatively few studies have been undertaken using metabolizable ions such as PO_4^{3-} (Lefebvre and Clarkson, 1984), NO_3^- (Presland and McNaughton, 1984; Lee and Clarkson, 1986; Siddiqi et al., 1991), SO_4^{2-} (Cram, 1983) and NH_4^+ (Macklon et al., 1990). Presland and McNaughton (1984) postulated the existence of four compartments (three in the roots and one in the shoot) based upon the distribution of ^{13}N among these tissues in maize plants. Using $^{15}NH_4^+$ efflux analysis with excised onion roots, the compartmental parameters for superficial, water free space, Donnan free space, cytoplasm and vacuole were identified (Macklon et al., 1990). The present study has characterized two intra-cellular compartments and one extra-cellular compartment for $^{13}NH_4^+$ in rice roots. The biochemical fractionation approach was also used to identify different compartments

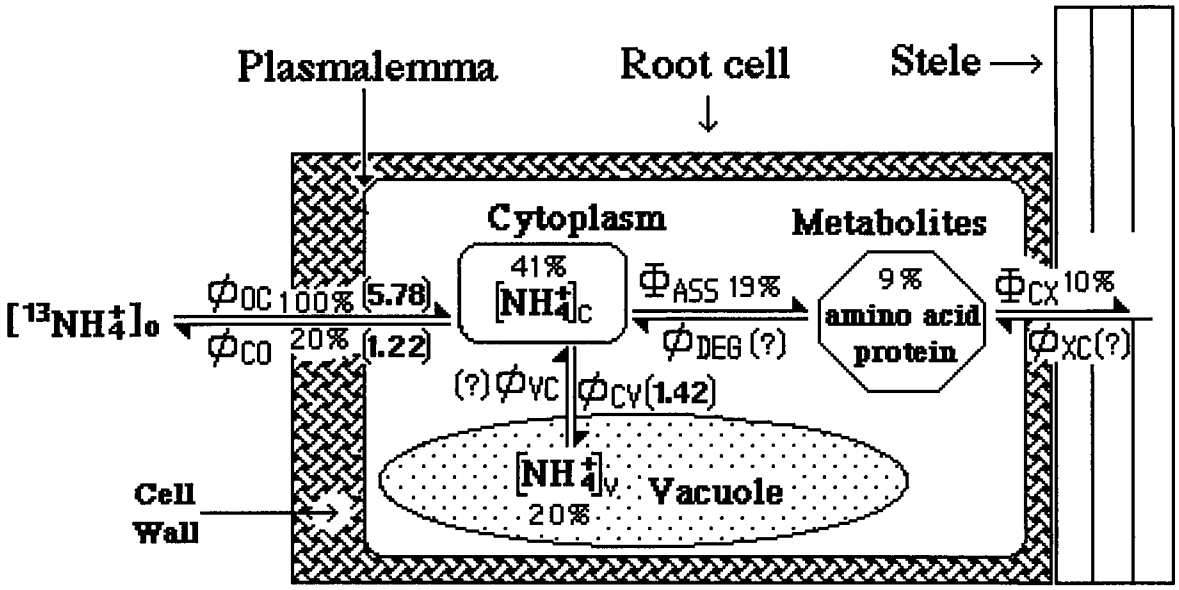


Figure 7. Proposed model for ammonium uptake and compartmentation in rice G100 roots. The bold values in parentheses are estimated fluxes of absorbed $^{13}NH_4^+$ (μmol $g^{-1}FW$ h^{-1}). The percentages represent the relative distributions of $^{13}NH_4^+$ among the compartments as a proportion of the isotope entering the cell during the 30 min loading. ϕ_{OC}, from outside plasmalemma to cytoplasm; ϕ_{CO}, from cytoplasm to outside plasmalemma; ϕ_{CV}, from cytoplasm to vacuole; ϕ_{VC}, from vacuole to cytoplasm; Φ_{CX}, metabolites translocation from root to shoot; ϕ_{XC}, metabolites translocation from shoot to root; Φ_{ASS}, assimilation rate; ϕ_{DEG}, degradation rate; ϕ represents chemical flux and Φ represents radioisotopic flux. Fluxes accompanied by (?) indicate fluxes for which data are not available from the present study.

for NH_4^+ assimilation. By using $^{15}NH_4^+$, three compartments were found corresponding to different cell types and a storage pool in barley roots (Fentem et al., 1983a) or different organelles (Rhode et al., 1980). Spatial differences in the activities of enzymes involved in NH_4^+ assimilation are also found along the root (Fentem et al., 1983a). In addition to this form of heterogeneity, there are distinct isozymes of glutamine synthetase, located within the cytosol and within plastids (Miflin and Lea, 1980).

Much less information is available concerning the partitioning of newly absorbed ammonium between these compartments, particularly concerning the partitioning between metabolized and un-metabolized fractions in the root and translocation to the shoot. In the present experiments, nearly 90% of absorbed ^{13}N remained in the roots, of which 80% was in the cation form ($^{13}NH_4^+$) after 30 min 'loading' (Table 6). Among the 'metabolized' ^{13}N pools (Φ_{ass}) in roots, significant quantities of absorbed ^{13}N (10%) were translocated to shoots (Φ_{cx}) during the experiment (Table 6), and analysis of this ^{13}N by ion-exchange chromatography (Table 1) revealed a virtual absence of $^{13}NH_4^+$. The remaining metabolized fractions consisted of 5.5% that failed to be held on the CEC, presumed to be amino acids and/or soluble protein, and 3.9%, which was not soluble and remained associated with the 'Root debris'. Calculations derived from results of both efflux and chemical analyses showed that un-metabolized NH_4^+ in the cytoplasm (Q_c) constituted only 8% of Q_i for G2 roots and ~30% for G100 and G1000 roots, respectively, (Table 4). Taking G100 plants as an example, a model describing the spatial and biochemical compartmentation of newly absorbed NH_4^+ uptake by rice roots is given in Fig. 7. About 24% of un-metabolized NH_4^+ was allocated to the cytoplasm and 76% to the vacuole. Based on the influx of $^{13}NH_4^+$ into

roots, 21% and 40% of ^{13}N remained in the cytoplasmic and vacuolar compartments, respectively, along with 20% that was effluxed and 19% that was assimilated. Of the 19% assimilated, roughly half (10% of influx) was translocated to shoots. This assimilation rate was based on total ^{13}N transported across the plasmalemma and may underestimate the true assimilation rate because during the loading period, the cytoplasmic $^{13}NH_4^+$ pool would not have reached steady state.

3.5. SUMMARY

Uptake of $^{13}NH_4^+$ by roots and distribution of $^{13}NH_4^+$ among plant parts and sub-cellular compartments was determined on rice plants grown hydroponically in MJNS containing 2, 100 or 1000 μM NH_4^+. At steady-state, the influx of $^{13}NH_4^+$ was determined to be 1.31, 5.78 and 10.11 μmol $g^{-1}FW\ h^{-1}$, respectively, for G2, G100 and G1000 plants; efflux was 11, 20, and 29%, respectively, of influx. The NH_4^+ flux to the vacuole was calculated to be between 1 to 1.4 μmol $g^{-1}FW\ h^{-1}$. By means of $^{13}NH_4^+$ efflux analysis, three kinetically distinct phases (superficial, cell wall, and cytoplasm) were identified, with half-lives for $^{13}NH_4^+$ exchange of 3 seconds, 1 and 8 minutes, respectively. Cytoplasmic [NH_4^+] was estimated to be 3.72, 20.55, and 38.08 mM for G2, G100 and G1000 plants, respectively. These concentrations were higher than vacuolar [NH_4^+], yet 72% to 92% of total root NH_4^+ was located in the vacuole. Distributions of newly absorbed $^{13}NH_4^+$ between plant parts and among the compartments were also examined. During a 30 minute period G100 plants metabolized 19% of the influxed $^{13}NH_4^+$. The remainder (81%) was partitioned among

the vacuole (20%), cytoplasm (41%) and efflux (20%). Of the metabolized ^{13}N, roughly one half was translocated to the shoots.

Chapter 4. KINETICS OF $^{13}NH_4^+$ INFLUX

4.1. INTRODUCTION

Despite the potential benefits of nitrate for the growth of rice plants, especially under anaerobic conditions (Malavolta, 1954; Bertani et al., 1986), ammonium is the predominant and most readily bio-available nitrogen form in paddy soil (Yu, 1985). It is the preferred nitrogen species taken up by rice (Fried et al., 1965; Sasakawa and Yamomoto, 1978), and in terms of the efficiency of fertilizer ultilization, ammonium is superior to nitrate in paddy soil (Craswell and Vlek, 1979).

Ammonium uptake systems have been well defined as concentrative, energy-dependent and carrier-mediated in algae (Smith and Walker, 1978), fungi (Kleiner, 1981), bacteria (Kleiner, 1985), and cyanobacteria (Boussiba and Gibson, 1991). However compared to the extensive investigations of NO_3^- uptake, the kinetics and energetics of ammonium transport in higher plants have received relatively little attention. In both rice plants and *Lemna* NH_4^+ uptake followed a bi-phasic pattern, with a saturable carrier-mediated system operating at low external NH_4^+ ($[NH_4^+]_o$) and either a second saturating system (Fried et al., 1965) or a linear diffusive component at elevated $[NH_4^+]_o$ (Ullrich et al., 1984). In N-starved *Lemna* both NH_4^+ uptake by the saturable system and depolarization of plasma membrane potential were found to exhibit the same concentration dependence (K_m's for both processes were 17 μM). At higher $[NH_4^+]_o$ the uptake by the linear system was not accompanied by further depolarization of membrane potential (Ullrich et al., 1984). The saturable

component of NH_4^+ uptake was sensitive to some metabolic inhibitors (Sasakawa and Yamamoto, 1978) and to changes of root temperature (Bloom and Chapin, 1981). In addition, NH_4^+ uptake is subject to negative feedback, supposedly from N metabolites (Lee and Rudge, 1986; Morgan and Jackson, 1989; Clarkson and Lüttge, 1991). Youngdahl et al., (1982) demonstrated that NH_4^+ uptake in rice decreased with plant age. However, despite these studies, the mechanism(s) of NH_4^+ uptake by roots of higher plants remain unclear. In particular, the high concentration system represents virtually unexplored territory.

Ammonium is unique among inorganic cations, because following absorption by plant roots, it is rapidly assimilated into organic pools. This has made the analysis of uptake and the subsequent fate of absorbed NH_4^+ much more complicated than for cations such as K^+ or Ca^{2+}. The availability of ^{13}N to this laboratory has enabled us to measure short-term $^{13}NH_4^+$ influx into roots of intact rice plants (Wang et al., 1991, 1993a, 1993b). This is critically important for two main reasons. Firstly, this technique allows determination of the particular flux (e.g. unidirectional plasma membrane influx or efflux), which is responding to the imposed conditions. By contrast, net uptake measurement, often obtained by means of long-term depletion experiments, actually measures the difference between influx and efflux. This is especially relevant because nitrogen (either NH_4^+ or NO_3^-) efflux has been reported to be significant, particularly at elevated concentrations of N (Morgan and Jackson, 1989; Breteler and Nissen, 1982; Wang et al., 1993). Secondly, by judicious choice of appropriate influx and desorption times, based upon the half-lives for exchange of the sub-compartments of the root (Lee and Clarkson, 1986; Presland and McNaughton, 1986; Siddiqi et al., 1991; Wang et al., 1993), it is possible to

measure the plasma membrane influx as opposed to other fluxes (to vacuole or to stele) which result from long-term experiments (Cram, 1968).

The objective of this study was to investigate the mechanisms and characteristics of ammonium uptake by rice plants. I have particularly emphasized short-term responses of $^{13}NH_4^+$ influxes to changes in $[NH_4^+]_o$ of uptake solutions over a wide range of external concentrations, in order to define the transport mechanisms responsible for influx across the plasma membrane. I have examined the influence of prior NH_4^+ provision upon the kinetic parameters for influx by both components of the bi-phasic system for NH_4^+ transport. In addition the sensitivities of these fluxes to metabolic inhibitors, short-term variations in temperature and pH were determined with a view to clarifying the mechanisms of these fluxes.

4.2. METHODS AND MATERIALS

4.2.1. Plant growth and ^{13}N production

See section 2.2. Seed germination; section 2.3. Growth conditions; section 2.4. Provision of nutrients; and section 2.5. Production of $^{13}NH_4^+$.

4.2.2. Relative growth rate

Rice seedlings were grown in 2, 100, and 1000 μM NH_4^+ (designated, hereafter, as G2, G100 and G1000 plants, respectively) to represent inadequate, adequate and excess nitrogen provision. Total fresh weights of

plants were recorded for three treatments at ages of 14, 21 and 28 d. They were used to calculate relative growth rates (RGR).

4.2.3. Influx measurement

See section 2.3.1.

4.2.4. Kinetic study

Influxes of G2, G100 or G1000 plants, respectively, were measured in ^{13}N-labeled MJNS varying in $[NH_4^+]_o$ from 2 μM to 40 mM in perturbation experiments. Perturbation experiments are defined as those in which plants are grown at one particular $[NH_4^+]_o$, and influxes are measured in a range of $[NH_4^+]_o$. Measured $^{13}NH_4^+$ influxes at various $[NH_4^+]_o$ were fitted to the Michaelis-Menten equation

$$V = (V_{max} \bullet [NH_4^+]_o)/(K_m + [NH_4^+]_o) \qquad [31]$$

and a more comprehensive equation

$$V = (V_{max} \bullet [NH_4^+]_o)/(K_m + [NH_4^+]_o) + b \bullet [NH_4^+]_o + a \qquad [32]$$

by means of a non-linear regression method using the computer program "Systat" (Wilkinson, 1987). In the equation, V (μmol $g^{-1}FW\ h^{-1}$) stands for the influx measured at a particular $[NH_4^+]_o$. V_{max} is the calculated maximum rate of influx while K_m (μM) represents $[NH_4^+]_o$ giving half of the maximum influx; b and a are constants characterizing the linear phase. At each concentration tested, influxes were determined in two to six separate

experiments with three or four replicates. Each replicate consisted of about 20 rice seedlings.

Based on the results of the kinetics studies (see Results), measured NH_4^+ influx from < 1 mM $[NH_4^+]_o$ appeared to result from a saturable high affinity transport system (hereafter referred to as HATS). Since the influx by the HATS had saturated between 0.1 and 1.0 mM $[NH_4^+]_o$, influx from 0.1 mM $[NH_4^+]_o$ was selected as a concentration representative of the HATS in the following studies. Above 1 mM $[NH_4^+]_o$, measured NH_4^+ influx appeared to result from the participation of both the HATS and a low affinity transport system (hereafter referred to as LATS). Therefore, the difference between measured influx at concentrations >1 mM $[NH_4^+]_o$ and the saturated values of the HATS were taken to represent fluxes due to the LATS.

4.2.5. Metabolic inhibitor study

Influxes were measured in MJNS containing representative levels of either 0.1 mM to estimate the activities of the HATS, or 20 mM NH_4Cl for the HATS plus LATS, in the presence or absence of different metabolic inhibitors. The inhibitors used were as follows: (1) 10 μM CCCP; (2) 1 mM CN^- plus SHAM; (3) 50 μM DES; (4) 0.1 mM DNP; (5) 50 μM Mersalyl; (6) 1 mM *p*CMBS. Details of preparation refers to Section 2.9.

In this study, both 3-week-old G2 and G100 plants were used. Before labeling with radioisotope, rice roots were treated with un-labeled MJNS containing the same concentrations of CN^- plus SHAM for 30 min. There were no pretreatments for the other inhibitors. Measurements of influx

were undertaken as in the kinetic study. Each inhibitor experiment was repeated twice with three replicates for each treatment. Each replicate consisted of about 20 seedlings. Therefore the means for influxes and standard errors were calculated from six replicates and represented the mean for approximately 120 seedlings.

4.2.6. Temperature study

Rice plants were grown under the same conditions as described previously, so that they were adapted to 20 ± 2°C. Influxes were subsequently measured in MJNS with either 0.1 mM or 20 mM NH_4Cl at solution temperatures of 5, 10, 20 and 30°C. During the pre-wash, uptake and post-wash, solutions were maintained at the designated temperatures. The measurements of influx were undertaken as in the kinetic study.

4.2.7. pH profile study

Rice plants were grown in MJNS containing 2 μM NH_4^+ under the conditions described in METHODS AND MATERIALS and adapted to growth medium at pH 6. Uptake solutions were adjusted to pH values of 3.0, 4.5, 6.0, 7.5 and 9.0 by additions of HCl or NaOH, respectively. To examine the effects of solution pH upon $^{13}NH_4^+$ influx, roots were exposed to the designated pH levels during 5 min pre-wash, 10 min influx as well as 3 min post-wash. Influxes of $^{13}NH_4^+$ were measured in either 0.1 mM or 10 mM NH_4^+ solution. The choice of 10 mM NH_4^+, rather than 20 mM was dictated by the desire to minimize additions of HCl or NaOH in adjusting pH levels in the uptake solutions.

4.3. RESULTS

4.3.1. Kinetics of $^{13}NH_4^+$ influx

Influxes of $^{13}NH_4^+$ in response to external concentrations in the range from 0.002 to 40 mM $[NH_4^+]_o$ were resolved into two distinct phases, presumably mediated by two separate transport systems; at low $[NH_4^+]_o$ (< 1 mM), a saturable high affinity transport system (HATS); and at high $[NH_4^+]_o$ (> 1 mM), the combined activities of a saturated HATS and a linear low affinity transport system (LATS).

4.3.2.1. HATS

In the low concentration range (< 1 mM $[NH_4^+]_o$), the values of $^{13}NH_4^+$ influx into roots of G2, G100 or G1000 rice plants conformed to Michaelis-Menten kinetics (Fig. 8). The kinetic parameters of V_{max} and K_m were estimated using non-linear regression analysis (Table 7) to fit the Michaelis-Menten equation. Analysis by means of a more comprehensive equation (see equation [32] in section 4.2.4.) gave similar trends although actual values of V_{max} and K_m were slightly different (data not shown). With increasing provision of NH_4^+ from 2, through 100 to 1000 μM in the period of two weeks prior to uptake measurements, root $[NH_4^+]_i$ increased from 2.37, through 4.31 up to 6.85 μmoles $g^{-1}FW$, respectively. As shown in Fig. 9, increasing $[NH_4^+]_i$ was associated with decreasing V_{max} values, from 12.8 through 8.2 down to 3.4 μmol $g^{-1}FW\ h^{-1}$, and increasing K_m values, from 32 through 90 up to 188 μM, for G2, G100 and G1000 plants, respectively.

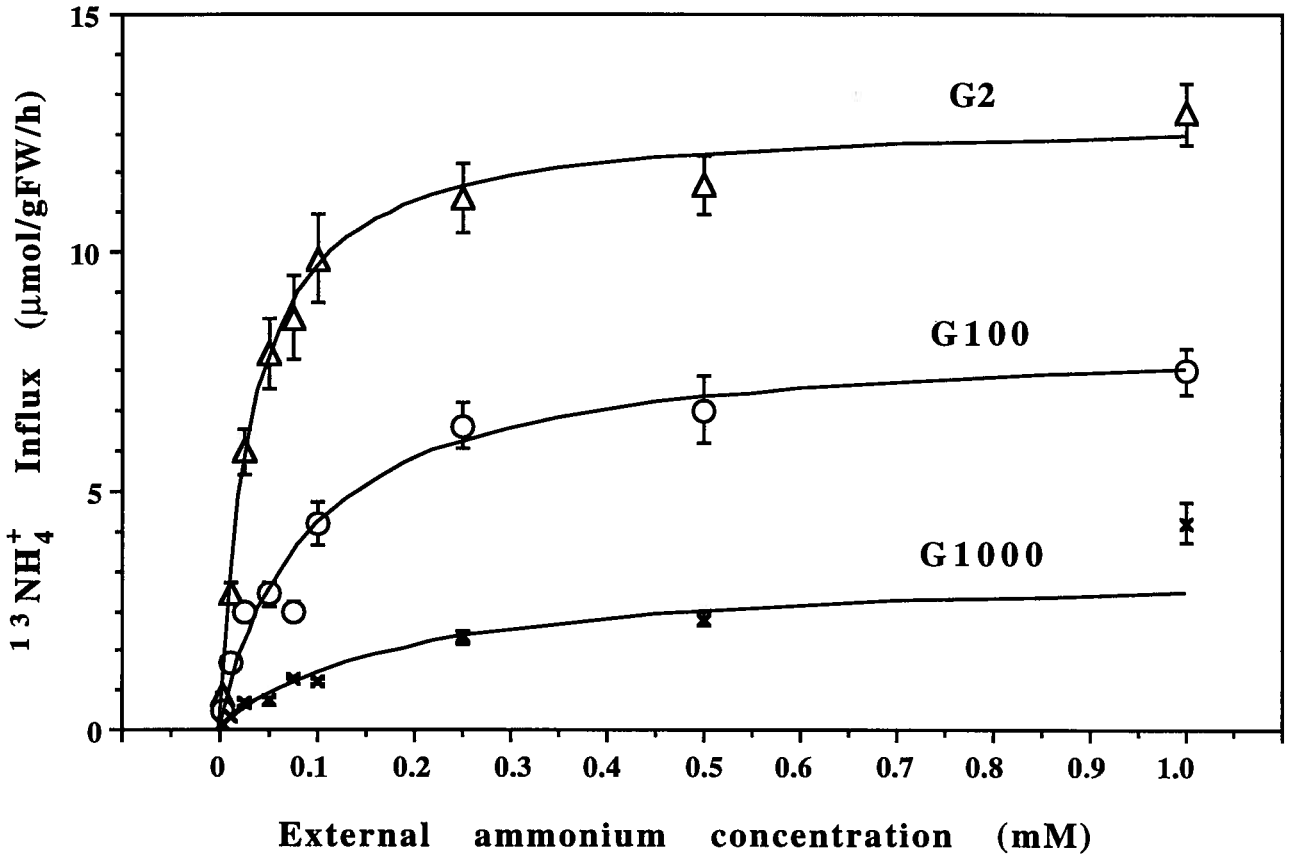


Figure 8. Concentration dependence of $^{13}NH_4^+$ influx at low $[NH_4^+]_o$. Influx of $^{13}NH_4^+$ into rice roots was measured in perturbation experiments. Rice seedlings were grown at 2, 100 or 1000 μM NH_4^+ (G2 (Δ), G100 (O) or G1000 (x), respectively). Each datum point is the mean of 16 replicates with standard error as a vertical bar. The solid lines are estimated from V_{max} and K_m values (Table 7) of G2, G100 and G1000 plants, respectively.

Table 7. Kinetic parameters for saturable and linear $^{13}NH_4^+$ influx of G2, G100 or G1000 roots as functions of $[NH_4^+]_o$. The relationships between $^{13}NH_4^+$ influx and $[NH_4^+]_o$ of uptake solution were estimated from Michaelis-Menten kinetics for influx measured between 2 to 1000 μM $[NH_4^+]_o$ and for linearity in the range of 1 to 40 mM, where 'a' is the intercept and 'b' is the slope.

		G2	G100	G1000
HATS [a]	V_{max}	12.8 ± 0.2 [b]	8.2 ± 0.7	3.4 ± 0.2
	K_m	32.2 ± 2.1	90.2 ± 23.2	188.1 ± 34.5
HATS+LATS	a	13.21	10.14	4.59
	b	0.67	0.79	1.30
	r^2	0.97	0.97	0.99
LATS	a	0.41	1.94	1.19
	b	0.67	0.79	1.30
	r^2	0.98	0.96	0.98

[a] HATS represents the high affinity transport system, measured below 1 mM $[NH_4^+]_o$. Influx measured at concentrations above 1 mM $[NH_4^+]_o$ is considered to be the combined contributions of both high and low affinity transport systems (HATS+LATS). LATS represents the low affinity transport system and is estimated by subtracting HATS from HATS+LATS. [b] V_{max} and K_m were estimated by non-linear regression with ± se.

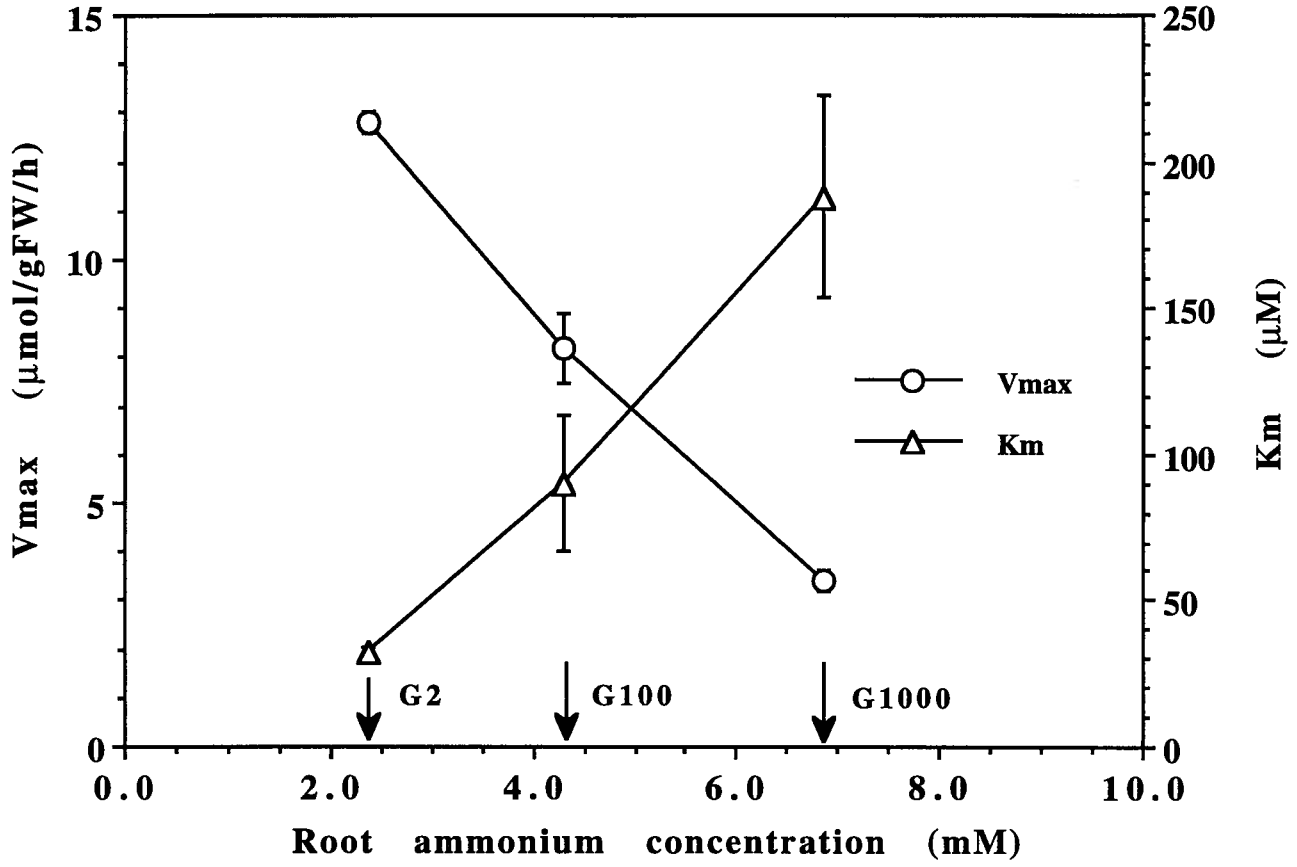


Figure 9. Relationship between kinetic parameters of NH_4^+ uptake and root ammonium concentrations ($[NH_4^+]_i$) of rice seedlings. The values of V_{max} (O) and K_m (Δ) from Fig. 8, were plotted against $[NH_4^+]_i$ for G2, G100, or G1000 plants, indicated by (↓) on the X axis.

4.3.1.2. LATS

In the higher range from 1 to 40 mM, the relationship between $[NH_4^+]_o$ and $^{13}NH_4^+$ influx was linear (Fig. 10A). The Y intercepts of these lines (13.21, 10.14 and 4.59 for G2, G100 and G1000, respectively) decreased according to the ammonium provision during the growth and agreed well with the corresponding V_{max} for the HATS (Table 7). Thus it is concluded that the measured fluxes at elevated $[NH_4^+]_o$ result from the combined activities of the HATS and the LATS. To evaluate the effect of prior NH_4^+ provision on the LATS for $^{13}NH_4^+$ influx without the influence of the HATS, the V_{max} values for HATS were subtracted from the measured influxes at elevated $[NH_4^+]_o$ values. The derived LATS values were re-plotted accordingly (Fig. 10B). As shown in Fig. 10B, $^{13}NH_4^+$ influx by LATS is higher for G1000 than for G100 or G2. Slopes of the lines increased according to the NH_4^+ level during growth period (0.67 for G2, 0.79 for G100 and 1.30 for G1000 in Table 7). These linear relationships at high $[NH_4^+]_o$ were confirmed by means of F-tests for linearity (Zar, 1974). Statistical analyses revealed that the slope of the G1000 line was significantly different from the slopes of the G2 and G100 lines (data not shown).

4.3.2. Effect of metabolic inhibitors on the influx of $^{13}NH_4^+$

In most cases $^{13}NH_4^+$ influxes of G2 plants were reduced by the presence of metabolic inhibitors in the uptake solutions as shown in Fig. 11. Net reductions of influxes, listed in Table 8, were calculated by using the influx of the control as zero reduction (0%). The HATS for NH_4^+ influx

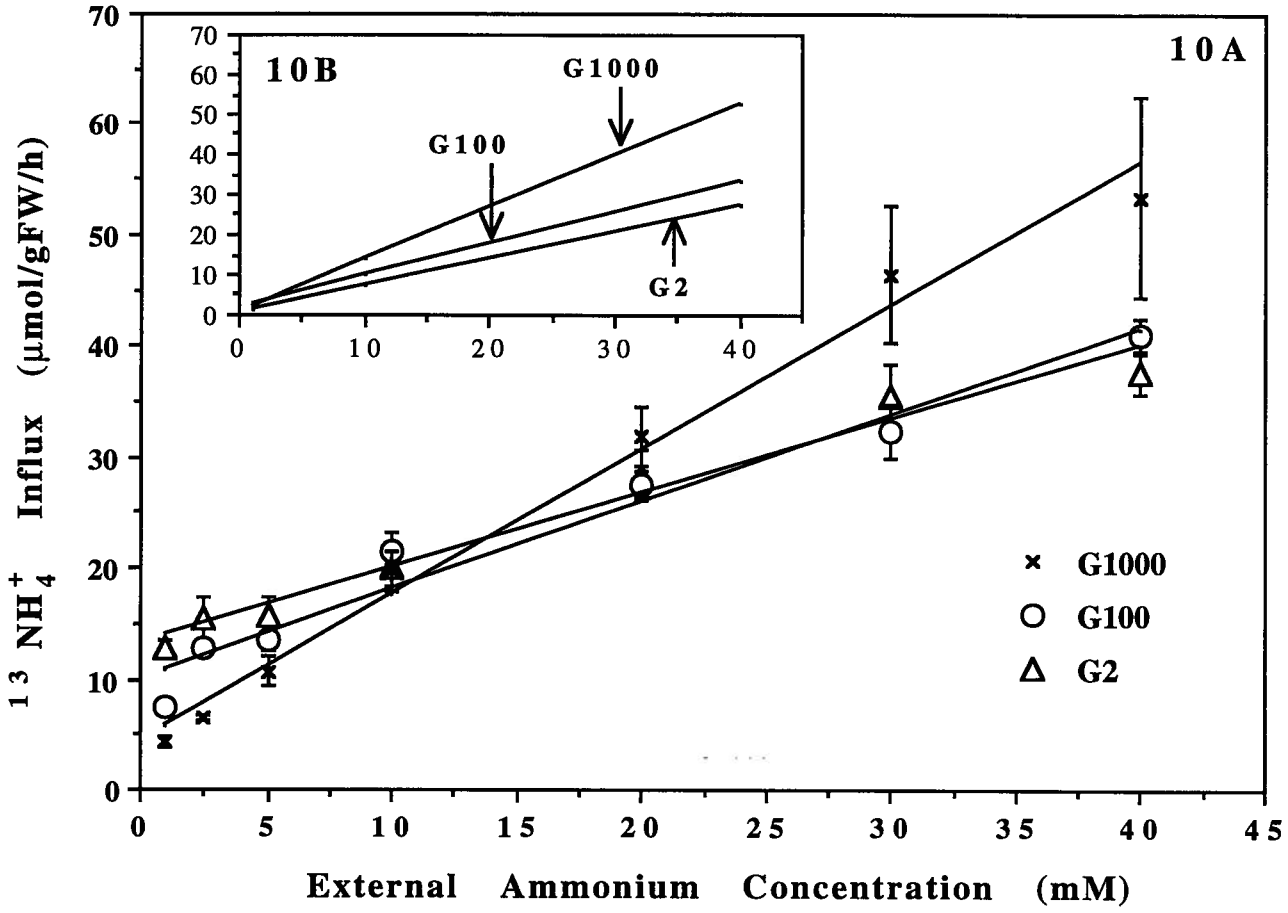


Figure 10. Influx of $^{13}NH_4^+$ into rice roots at high $[NH_4^+]_o$ in perturbation experiments. **10A:** Influxes of $^{13}NH_4^+$ into G2 (Δ), G100 (O), or G1000 (×) roots, respectively, were plotted against $[NH_4^+]_o$. Each datum point is the mean of more than 6 replicates with ± se as vertical bar. **10B:** The estimated LATS Fluxes after subtracting the V_{max} of the HATS of G2, G100 or G1000, respectively, from the corresponding measured influxes (in 9-A). These plotted lines of LATS have the same slopes as their corresponding lines in 9-A but with slightly different values of the intercept, 0.53, 1.96 and 0.99 for G2, G100, and G1000 plants, respectively.

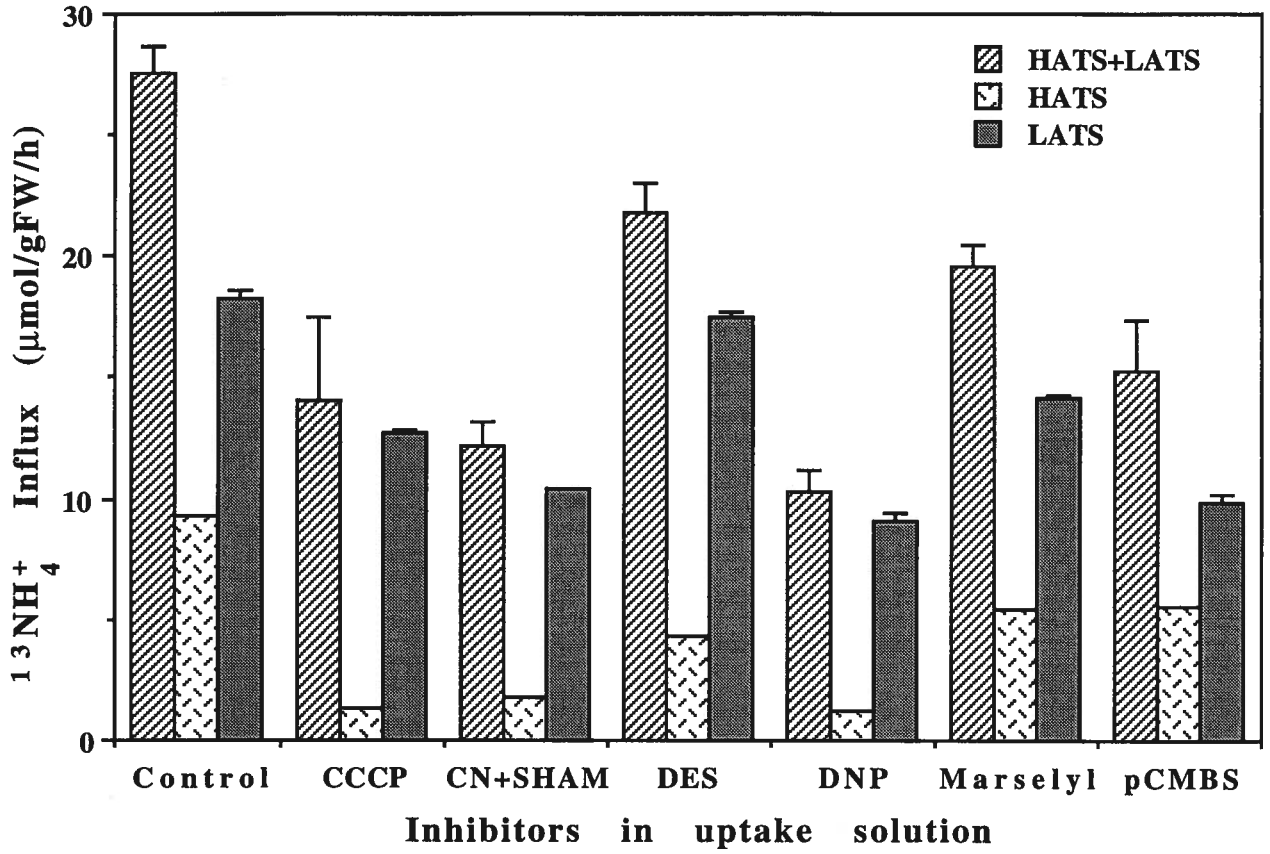


Figure 11. Effect of metabolic inhibitors on $^{13}NH_4^+$ influx. Rice plants were grown in MJNS containing 2 μM NH_4Cl. Influxes of $^{13}NH_4^+$ were measured in MJNS with either 0.1 mM or 20 mM NH_4^+ in the presence or absence of a specific metabolic inhibitor. Each datum point is the average of more than 6 replicates with standard error as vertical bar. Abbreviations: CCCP (10 mM): Carboxylcyanide m-chlorophenylhydrazone; CN^- plus SHAM (1 mM): NaCN and salicylhydroxamic acid; DES (50 mM): diethylstilbestrol; DNP (0.1 mM): 2,4-dinitrophenol; Mersalyl (50 mM): mersalyl acid; *p*CMBS (1 mM): *p*-chloromercuri-benzenesulfonate.

was reduced by 81 to 87% by the protonophore (CCCP) or the un-coupler of electron-transport-chain (CN^- plus SHAM) and inhibitors of ATP synthesis (DNP). These three treatments reduced the LATS by only 31 to 51%. ATPase inhibitor DES reduced $^{13}NH_4^+$ influx due to the HATS by 51% but had negligible effects on LATS. External protein modifiers of the membrane surface, *p*CMBS and Mersalyl, reduced $^{13}NH_4^+$ influx of HATS by about 40% with slightly less or similar reductions of LATS (22 to 46%). These patterns of inhibition were also observed for G100 plants (data not shown).

4.3.3. Effect of root temperature on $^{13}NH_4^+$ influx

Short-term perturbations of root temperature significantly affected the influx of $^{13}NH_4^+$ into rice roots that were adapted to the growth temperature of 20°C (data not shown). Table 9 shows the calculated Q_{10} values for G2 and G100 plants in the temperature range from 5°C to 30°C. In this temperature range the Q_{10} values for HATS fell from > 2.4 between 5 to 10°C to 1.25 between 20 to 30°C. The results of F-tests in conjunction with Duncan's Multiple Range Tests demonstrated that Q_{10} values for the different temperature ranges were significantly different for the HATS ($P >$ 0.05). In contrast, there were no significant differences between the Q_{10} values for LATS in the same three temperature ranges for both G2 and G100 plants ($P > 0.05$). Nevertheless Q_{10} values for the LATS were significantly greater than 1.

Table 8. Reduction of $^{13}NH_4^+$ influx into roots of G2 plants by various metabolic inhibitors.

Treatment	Inhibitor Level	% Reduction of	
		HATS [a]	LATS [b]
Control	None	0	0
CCCP	10 mM	84.58	30.72
CN+SHAM	1 mM	80.84	43.20
DES	50 mM	53.96	4.00
DNP	0.1 mM	86.72	50.55
Mersalyl	50 mM	41.97	22.40
*p*CMBS	0.5 mM	41.33	46.11

[a] The influxes of HATS were measured in the representative $[NH_4^+]_o$ (0.1 mM). Reduction of HATS (%) was calculated by setting the 'Control' value, the reduction of influx value measured in 0.1 mM NH_4Cl uptake solutions without the inhibitor, as 0%.

[b] Reduction of LATS (%) was calculated by first determining the influx due to LATS by subtracting the influx values measured at 0.1 mM NH_4^+ from that at 20 mM NH_4^+ for control and for each inhibitor treatment, respectively. The reduction of influx value due to LATS under control conditions was then set at 0%.

Table 9. Calculated Q_{10} values for $^{13}NH_4^+$ influx by the HATS or LATS of rice plants grown at 20°C with 2 or 100 μM NH_4Cl (G2 and G100 plants).

	Temperature Range	G2 Plants [a]	DMRT [b]	G100 Plants	DMRT
(a) HATS :	5 - 10°C	2.48 ± 0.04	a	2.59 ± 0.21	a
	10 - 20°C	1.79 ± 0.08	b	1.68 ± 0.22	b
	20 - 30°C	1.25 ± 0.16	c	1.44 ± 0.16	b
(b) LATS [c] :	5 - 10°C	1.41 ± 0.21		1.54 ± 0.27	
	10 - 20°C	1.49 ± 0.06		1.90 ± 0.46	
	20 - 30°C	1.56 ± 0.06		1.33 ± 0.12	

[a] Each value (± se) is the average of three means from duplicate experiments; each mean is derived from three replicates. [b] DMRT stands for Duncan's Multiple Range Test for comparing all possible pairs of treatment means. Means having a common letter are not significantly different at the 5% significance level. [c] Both F-tests and DMRT indicated that means for the LATS were not significantly different at the 5% level.

4.3.4. Effect of solution pH on $^{13}NH_4^+$ influx

The effect of uptake solution pH on $^{13}NH_4^+$ influx was also investigated. The percentage of the control was computed on the basis of the influx value at pH 6.0 for either HATS or LATS (Table 10). In this case, a Least Significant Difference test (LSD) was used for making pairwise comparisons between the control and other treatments. In the range from 4.5 - 9.0, solution pH had only a small effect on $^{13}NH_4^+$ influx from 0.1 mM $[NH_4^+]_o$, whereas $^{13}NH_4^+$ influx by LATS decreased very significantly with increasing ambient pH beyond pH 6.0. By contrast, reduction of solution pH down to 3.0 drastically reduced $^{13}NH_4^+$ influx by HATS as well as LATS.

4.4. DISCUSSION

4.4.1. Kinetics of ammonium uptake

In Chapter 3 and in Wang et al., (1993a) it was demonstrated that the half lives for $^{13}NH_4^+$ exchange of the cell wall and cytoplasmic phases of rice roots (G2, G100 or G1000 plants) were approximately 1 and 8 min, respectively (Section 3.3.1., Table 2). By using 10 min exposures to $^{13}NH_4^+$ and 3 min post-washes, therefore, estimates of plasma membrane influxes rather than net flux or quasi-steady fluxes to vacuole were obtained (see Cram, 1968). The results of the present study revealed that NH_4^+ influx across the plasma membrane into rice roots exhibits a bi-phasic pattern: in the low range (below 1 mM $[NH_4^+]_o$), influx occurred via a saturable high affinity transport system (HATS); while from 1 to 40 mM $[NH_4^+]_o$ a second, low affinity, non-saturable transport system (LATS) became apparent. This

Table 10. Effect of uptake solution pH on $^{13}NH_4^+$ influx into rice roots of 3-week-old G2 plants grown at pH = 6.0 in MJNS. Influx of $^{13}NH_4^+$ was measured in MJNS at various pH levels (3.0, 4.5, 6.0, 7.5, and 9.0) with $[NH_4^+]_o$ at either 0.1 mM for the HATS or 10 mM for the HATS+LATS. The value of LATS was obtained by subtracted the values of HATS from HATS+LATS of each treatment.

	pH	Influx [a]	LSD [b]	(%) of Control [c]
(a) HATS :	3.0	6.91 ± 1.43	*	53
	4.5	12.02 ± 0.46	ns	87
	6.0	13.22 ± 0.27	control	100
	7.5	14.51 ± 0.39	ns	109
	9.0	12.94 ± 0.30	ns	95
(b) LATS :	3.0	15.75 ± 0.45	*	87
	4.5	18.63 ± 2.80	ns	103
	6.0	18.07 ± 0.49	control	100
	7.5	11.44 ± 1.37	*	63
	9.0	9.29 ± 1.54	*	51

[a] Each value (± se) (μmol $g^{-1}FW\ h^{-1}$) is the average of four means of duplicate experiments. Each mean is derived from three replicates. [b] LSD stands for Least Significant Difference test, used for making pairwise comparisons between the control at pH 6.0 and other treatments. * = significant at 5% level and ns = not significant. [c] The percentages of control were calculated using the NH_4^+ influx measured at pH=6.0 as 100%.

bi-phasic pattern of uptake has been reported for NH_4^+ uptake by *Lemna* (Ullrich et al., 1984), for K^+ uptake by corn roots (Kochian and Lucas, 1982), and for NO_3^- uptake by barley roots (Siddiqi et al., 1990).

Plasma membrane $^{13}NH_4^+$ influx at low $[NH_4^+]_o$ conformed to Michaelis-Menten kinetics (Table 7) in accord with earlier studies of net NH_4^+ uptake by rice (Youngdahl et al., 1982; Wang et al., 1991). This has also been found to be the case for roots of other species, including corn (Becking, 1956), rye-grass (Lycklama, 1963), and barley (Bloom and Chapin, 1981), where net NH_4^+ uptake rates saturated in the range from 100 to 1000 μM $[NH_4^+]_o$. The significance of this HATS for NH_4^+ in rice roots is that it allows plants to absorb sufficient nitrogen (NH_4^+) from very low levels in the rhizosphere to meet the minimum requirement for plant growth. In the present experiments, for example, by three weeks, the relative growth rates were independent of $[NH_4^+]_o$ from 100 to 1000 μM NH_4^+. The relative growth rates calculated from total fresh weight of both G100 and G1000 plants were at ~0.16 d^{-1} for the third week of growth while for G2 the value was ~0.06 d^{-1}. By the fourth week the differences in RGR had diminished to 0.05, 0.06, and 0.06 d^{-1}, respectively for G2, G100 and G1000 plants. The reduced growth rates of G2 plants were accompanied by increased root:shoot ratios, and leaves were slightly paler than those of plants grown at higher $[NH_4^+]_o$.

At the higher range of $[NH_4^+]_o$ (1 to 40 mM), a linear, low affinity transport system (LATS) also participated in NH_4^+ uptake by rice roots, as is the case for other ions and plant species (Kochian and Lucas, 1982; Ullrich et al., 1984; Pace and McClure, 1986; Siddiqi et al., 1990). The Y intercepts for lines of measured influx (due to both transport systems) against $[NH_4^+]_o$ were in good agreement with the corresponding V_{max}

values for the HATS (Table 7), which suggests that the two distinct transport systems (HATS and LATS) are additive.

Despite the importance of NH_4^+ as principal source of N for many plant species and the increasing availability of techniques for the measurement of short-term $^{13}NH_4^+$ and $^{15}NH_4^+$ influxes, few detailed influx isotherms (as distinct from net uptake isotherms) have been reported for NH_4^+ influx into roots of higher plants. Nevertheless, Ullrich et al., (1984) were able to demonstrate linear kinetics of NH_4^+ uptake by *Lemna* between 0.1 to 1.0 mM $[NH_4^+]_o$ using a depletion method. The question of the saturation of this apparently linear system at higher concentrations remained unresolved. Clearly, it is difficult to measure net fluxes by employing concentration depletion methods at high external concentrations without extending the uptake experiment for long periods of time. By using short-lived radioisotopes, such as ^{13}N, it has been possible to measure unidirectional fluxes of NO_3^- and NH_4^+ at the plasma membrane of intact plant roots (Glass et al., 1985; Ingemarson, 1987; Presland and McNaughton, 1986; Lee and Clarkson, 1986; Siddiqi et al., 1990; Wang et al., 1993). Even at concentrations as high as 40 mM, there was no evidence of saturation of the LATS system (Fig. 10).

4.4.2. Energetics of ammonium uptake

The influx of ammonium by HATS is clearly dependent on metabolic energy. In the present study metabolic inhibitors, CCCP, DNP or CN^- plus SHAM, diminished $^{13}NH_4^+$ influxes of HATS by more than 80% (Table 8). The effects of these inhibitors on the LATS were much smaller (31 to 51% inhibition). Further evidence from the Q_{10} values (Table 9) supported the

notion of energy dependence. A Q_{10} value greater than 2 is considered to indicate the metabolic dependence of physiological processes such as ion transport. Short-term perturbations of temperature between 5 to 10°C, significantly increased the Q_{10} values for HATS up to ~ 2.5 compared to ~1.5 between 20 to 30°C. In a 7 h concluded that the uptake of ammonium by 9-day old rice seedlings was closely associated with metabolism. However, such long-term studies probably measure the Q_{10} for NH_4^+ assimilation rather than the transport process. The values of Q_{10} estimated from Ta and Ohira's data (1982) provided values larger than 2.5 for $^{15}NH_4^+$ absorption by rice roots between 9 to 24°C. Lower Q_{10} values (1.0 to 1.6) were reported for net ammonium uptake of low-temperature adapted ryegrass (Clarkson and Warner, 1979); barley (Bloom and Chapin, 1981); and oilseed rape (Macduff et al., 1987) indicating that NH_4^+ transport had acclimated to the low temperature growth conditions. Consistent with the results of the metabolic inhibitor studies, the present Q_{10} study indicated that LATS was less sensitive to changes of root temperature than the HATS (Table 9).

The apparent energy-dependence of the HATS may not necessarily mean that NH_4^+ uptake is an active transport process, although active transport systems for ammonium have been proposed in bacteria, fungi and algae (Kleiner, 1981; Schlee and Komor, 1986, Singh et al., 1987). The accumulation of NH_4^+ against its concentration gradient could be achieved by active or passive uptake mechanisms: the former, by direct use of metabolic energy to carry a solute across a membrane towards a region of higher electrochemical potential; while the latter, by solute flux across a membrane along the electrochemical potential gradient, a process that may be only indirectly related to metabolic energy.

According to the compartmental analysis (Chapter 3 and in Wang et al., 1993a), the cytoplasmic concentration of NH_4^+ in G2 roots was estimated to be 3.7 mM. Using this value and -130 mV as measured plasma membrane membrane electrical potential difference for G2 plants in 'MJNS' minus Nitrogen solutions (Wang et al., 1992), predictions derived from the Nernst equation indicated that net ammonium uptake would be active only when $[NH_4^+]_o$ falls below 125 μM. This is rather similar to the value of 67 μM calculated for *Lemna* (Ullrich et al., 1984). However, this calculation only serves to predict the feasibility of the process occurring under the prescribed conditions. The precise relationship between the calculated electrochemical potential difference for an ion and the putative transport systems, predicted on the basis of concentration-dependent influx curves, are difficult to realize. In the present case, for example, there are no discontinuities in the uptake curve corresponding to the predicted concentration at which the switch between active and passive transport (~125 μM $[NH_4^+]_o$) occurs. This issue is raised to warn against a too literal interpretation of the thermodynamic predictions. While on thermodynamic grounds influx is uphill below 125 μM and downhill beyond this level, the kinetic data reveal no apparent change of transport mechanism.

The characteristics of the two transport systems for NH_4^+ influx have significant features in common with those described for K^+ uptake in which (incidentally) there is yet no clear consensus regarding the mechanisms of influx into higher plant roots. Likewise, the mechanism of the apparently active transport of ammonium below 125 μM is unknown. It might occur by means of a specific ATPase or a secondary transport system such as an NH_4^+:H^+ symport that is driven by the proton motive force (pmf). As

proposed for K^+ uptake by *Neurospora*, for each K^+ entering, one H^+ is co-transported and $2H^+$ are extruded by the proton pump (Rodriquez-Navarro et al., 1986). The net result is therefore a 1:1 K^+/H^+ exchange. Is it possible that NH_4^+ influx is mediated by an analogous system? It has long been documented that NH_4^+ uptake is associated with strong acidification of the external medium (e.g. Becking, 1956). Likewise in the present study, when pH was not adjusted daily in the initial growth experiments, external pH dropped so low that plants failed to grow normally.

So far as the passive uptake of ammonium is concerned at higher concentrations, several authors have proposed that NH_4^+ influx may occur by an electrogenic uniport in response to the electrical gradient (Kleiner, 1981; Ullrich, 1984). When ambient concentration is beyond the predicted threshold for active uptake, the concentrative NH_4^+ uptake may be due to a facilitated transport system driven by the electrochemical potential difference for NH_4^+. This has two components; the difference in chemical potential of NH_4^+ ($\Delta\mu_{NH_4^+}$) between cytoplasm and outside and the electrical potential difference ($\Delta\Psi$) generated in part by proton efflux across the transducing membrane. The actual mechanistic link, if one exists, between NH_4^+ influx and the pmf across the plasma membrane is unclear at present. Certainly the results of the treatments with the protonophore (CCCP) or the un-coupler of ATP formation (DNP and CN^- plus SHAM), which caused greater than 81% reduction of influx due to HATS, are consistent with a dependence of NH_4^+ influx on transmembrane pmf. Further support for this hypothesis is provided by the effect of ATPase inhibitor, DES, which reduced $^{13}NH_4^+$ influx due to HATS by 54% but had negligible effects on LATS.

4.4.3. Effect of pH profile on ammonium uptake

In the present study, influx by the HATS was strongly reduced below pH 4.5. By contrast, in the range from pH 4.5 to 9.0, $^{13}NH_4^+$ influx by the HATS appeared to be relatively insensitive to pH. $^{13}NH_4^+$ influx by the LATS actually decreased with increasing ambient pH beyond pH 6.0. It has been reported for several species that the specific uptake rate of NH_4^+ can be reduced by short-term decreases in pH below 6.0 (Munn and Jackson, 1978; Marcus-Wyner, 1983; Vessey, 1990) and even terminated all-together at pH 4.0 (Tolly-Henry and Raper, Jr., 1986). Tanaka (1959) suggested that rice is very sensitive to pH below 4. Most probably this reflects a general detrimental effect of such acidic conditions on the transport systems. In addition, it has been observed that when plants were grown at such low pH values over extended periods of time, the roots became stunted and discolored. It has been suggested that both high pH and/or high ammonium concentration of solution may result in high rates of NH_3 uptake due to increased NH_3 concentration and the higher permeability of cell membranes to NH_3 than NH_4^+ (see Macfarlane and Smith, 1982). However, in many studies this expectation has not been observed, and uptake failed to increase at elevated pH (MacFarlane and Smith, 1982; Deane-Drummond, 1984; Schlee and Komor, 1986). Likewise, in the present study, influxes of $^{13}NH_4^+$ due to the LATS were reduced by 25 - 35% at higher pH (7.5 - 9.0), despite a predicted increase of $[NH_3]$ from less then 0.1% of total $[NH_4^+ + NH_3]$ at pH 6.0, to 36% at pH 9.0 according to the pK_a for NH_4^+ (9.25). Furthermore, membrane electrical potentials of rice roots have been shown to be depolarized by elevated ammonium concentrations (Wang et al., 1992). These observations indicate the entry of cation (NH_4^+) rather than neutral ammonium (NH_3). The

evidence from our electrophysiological study of rice roots indicated a linear relationship between depolarization of membrane potential and influx of NH_4^+ from 1 to 40 mM (data not shown). Therefore, at elevated concentration and pH, it is unlikely that simple diffusion of NH_3 could be considered as a major component of the influx of LATS. Nevertheless, in their study using *Lemna*, Ullrich et al., (1984) reported that depolarization of membrane potential was saturated at ~ 0.1 mM even though net uptake continued to 1 mM in a linear pattern. This observation is consistent with NH_3 entry by the LATS in *Lemna*.

4.4.4. Regulation of ammonium uptake

Although the bi-phasic pattern of NH_4^+ influx was independent of the prior NH_4^+ exposure, the individual systems, particular the HATS, were extremely sensitive to prior NH_4^+ exposure (Figs. 8, 9, 10). Evidently NH_4^+ influx by the HATS was subject to regulation by negative feedback: with increasing $[NH_4^+]_o$ in the growth medium, root $[NH_4^+]_i$ increased and NH_4^+ influx decreased (Fig. 9). It is noteworthy that in the present case, negative feedback regulation appeared to affect both V_{max} and K_m values (Table 7, Figs. 8 and 9). It has commonly been observed that V_{max} is strongly and unequivocally influenced by the level of nutrient supplied during growth. By contrast, an effect on K_m has rarely been observed (Lee, 1982). Only in the case of K^+ (Glass, 1976) was the K_m strongly influenced by K status although other ions such as Cl^- do show small changes (Lee, 1982). In the present study, the values of K_m were strongly influenced by the prior level of NH_4^+ supply, and are positively correlated with $[NH_4^+]_i$.

Contrary to expectation, $^{13}NH_4^+$ influxes due to the LATS were higher in plants previously maintained at 1000 μM NH_4^+ than in those maintained at 2 μM NH_4^+. The reverse was found to be the case for $^{13}NO_3^-$ influx in barley (Siddiqi et al., 1990). This positive correlation between provision of NH_4^+ and $^{13}NH_4^+$ influxes at high $[NH_4^+]_o$ may indicate that the LATS may not be subject to regulation by negative feedback. Another possible explanation is that better nitrogen nutrition may provide more building materials (protein?) for constructing transporters. However, exposures to high $[NH_4^+]_o$ (>1 mM) were brief and in longer exposures NH_4^+ influx may be down-regulated in accord with expectation.

The present study has demonstrated the strong negative down-regulation of influx by the HATS in response to elevated NH_4^+ supply during growth. At present the mechanism(s) and signals responsible for this down-regulation of uptake are unclear. Feedback signals may result from un-metabolized ammonium of root cells or reduced nitrogen (Lee, 1982; Morgan and Jackson, 1989). Lee and Rudge (1986) have suggested that in barley the uptake of NH_4^+ and NO_3^- are under common negative feedback control from a product of NH_4^+ assimilation rather than NH_4^+ and/or NO_3^- accumulation *per se*. Reduced N pools which cycle in xylem and phloem from root to shoot have been implicated in the whole plant regulation of N uptake by plant roots (Cooper and Clarkson, 1989). However, Siddiqi et al. (1990) have suggested that in the case of NO_3^- influx, vacuolar accumulation of NO_3^- *per se* may also, at least indirectly, participate in flux regulation. Further support for this proposal has come from studies of nitrate reductase mutants of barley that are capable of normal induction of NO_3^- uptake and appear to show diminished $^{13}NO_3^-$ influx as NO_3^- accumulates (King et al., in press). In the present study, also,

there was a close negative correlation between NH_4^+ influx and $[NH_4^+]_i$ in root tissues (Fig. 9). However, the altered NH_4^+ status in G2, G100, and G1000 plants was probably also associated with changes in organic N fractions. Since efflux was estimated to be 10 to 30% of influx for G2, G100 and G1000 plants, respectively (Wang et al., 1993), negative feedback acts very strongly on the influx step of the HATS, but since efflux also increased with increasing $[NH_4^+]_o$, this flux will exert significant effects upon net uptake.

4.5. SUMMARY

The work described provides the first detailed characterization of NH_4^+ influx across the plasma membrane of rice roots. Ammonium influx is bi-phasic, mediated by two discrete transport systems. Metabolic inhibitor studies and Q_{10} determinations indicated that both systems were energy-dependent, although the HATS consistently showed greater sensitivity to metabolic interference than the LATS. Nevertheless, thermodynamic evaluations indicate that only at quite low $[NH_4^+]_o$ is there a need to invoke active transport of NH_4^+ against the electrochemical gradient. It is highly unlikely that the LATS is active. The HATS was found to be extremely sensitive to prior exposure to ammonium as indicated by the altered values of K_m and V_{max}. General insensitivity of influx to pH in the range from 4.5 to 9.0 argues strongly against significant entry of NH_3 across the plasma membrane even at high $[NH_4^+]_o$.

Chapter 5. ELECTROPHYSIOLOGICAL STUDY

5.1. INTRODUCTION

Ammonium influx by rice roots (*Oryza sativa* L. cv. M202) has been shown to exhibit a biphasic dependence on $[NH_4^+]_o$ (Wang et al., 1991, 1992b; 1993b). At low $[NH_4^+]_o$, influx is mediated by a saturable HATS which exhibits high Q_{10} values between 10 and 30 °C and a significant sensitivity to metabolic inhibitors (Wang et al., 1993b). At elevated $[NH_4^+]_o$ (between 1 and 40 mM), NH_4^+ influx increases in a linear fashion with increasing $[NH_4^+]_o$, and though still exhibiting energy-dependence, this LATS was shown to be less responsive to metabolic inhibitors (Wang et al., 1993b). A biphasic pattern of NH_4^+ uptake of this sort, with both saturable and linear phases, was first reported in *Lemna*, by Ullrich et al., (1984).

In order to make a definitive evaluation of the thermodynamics of NH_4^+ influx (passive versus active transport), it is essential to determine the chemical potential difference for NH_4^+ between the cytoplasm and external media, and $\Delta\Psi$ across the plasma membrane. In Chapter 3, compartmental analysis was used to estimate cytoplasmic $[NH_4^+]$. So far as I am aware, only one report measuring $\Delta\Psi$ in rice roots has appeared in the literature: Usmanov (1979) reported $\Delta\Psi$ to be -160 mV. As early as 1964, Higinbotham et al. noted the marked depolarizing effect of $[NH_4^+]_o$ on coleoptile cell $\Delta\Psi$ in oats. Likewise, Walker et al. (1979a, b) demonstrated the transport of ammonium and methylamine across the plasma membrane of *Chara*, and the depolarizing effects of these cations. The most

detailed study of the concentration dependence of $\Delta\Psi$ depolarization by NH_4^+ was undertaken by Ullrich et al. (1984), using *Lemna*. Below 0.2 mM $[NH_4^+]_o$ both NH_4^+ uptake and $\Delta\Psi$ depolarization responded in a saturable fashion with half-saturation values of 17 μM for both processes. From 0.2 to 1 mM , net uptake of NH_4^+ responded linearly to $[NH_4^+]_o$, with no further $\Delta\Psi$ depolarization. On the basis of this observation, Ullrich et al. (1984) concluded that the linear system might result from diffusion of NH_4^+ or NH_3 across the plasma membrane.

The present study was initiated, therefore, to estimate $\Delta\Psi$ in intact rice roots, under conditions corresponding to those employed to estimate cytoplasmic $[NH_4^+]$ in our previous study, and to determine the concentration dependence of the depolarizing effect of $[NH_4^+]_o$. The effects of metabolic inhibitors on $\Delta\Psi$ were also examined.

5.2. Materials and Methods

5.2.1. Growth of plants

Rice (*Oryza sativa* L. cv. M202) seeds were surface sterilized in 1% NaOCl for 30 min and rinsed with deionized water. Seeds were imbibed overnight in aerated deionized water at 38°C before planting on plastic mesh mounted on the bottoms of polyethylene cups. Four cups (3 to 4 seeds per cup) were set in the lid of a 1-L black polyethylene vessel with the solution level just above the seeds. Seeds were allowed to germinate in the dark (at 20°C) for 4 days. At day 5, rice seedlings were exposed to light and MJNS containing the designated levels of NH_4Cl. The composition of

MJNS, growth conditions, nutrient supply and pH adjustment were those described in Section 2.1.2. The growth medium in the 1-litre polyethylene vessels were completely replaced on alternate days and the nutrient levels were topped up with concentrated stock solutions daily. Rice plants used in the experiments were 3-week-old G2 or G100 plants respectively.

5.2.2. Measurements of cell membrane potential

Plasma membrane $\Delta\Psi$ of rice roots were measured as described by Kochian et al. (1989) and Glass et al. (1992). In short, rice plants were secured in the larger part of a flow-through Plexiglas impalement chamber, and one intact root was carefully placed over the platinum pins in a narrow section of the chamber. This root was held firmly during the impalement by two short lengths of Tygon tubing, from each of which a small wedge had been cut. The tubing was placed on either side of the impalement zone to clamp the root in place. All impalements were made in a region about 1 to 3 cm behind the root tip, using a hydraulically driven, three-dimensional micromanipulator (Model MO-20, Narashige, USA). Both the Plexiglas impalement chamber and micromanipulator were mounted on the microscope stage. Microelectrodes (including impaling, reference and grounding electrodes) were made from 1.0 mm single-barreled borosilicate glass tubing pulled to a tip diameter of ~0.5 μM and filled with 3M KCl (adjusted to pH 2 to reduce tip potentials). Measured membrane potentials of root cells, which are the voltage differences between the impaling and reference electrode, were amplified and recorded on a strip chart recorder. During impalement, solutions were continuously delivered from an air-pressured reservoir to the chamber through tygon tubing at controlled flow rates (~7.5 ml min^{-1}).

5.2.3. Experimental treatments

At the beginning of each experiment, the impalement was made on G2 or G100 roots bathed in their growth media (MJNS containing 2 or 100 μM NH_4Cl, respectively) and the membrane potential was recorded ($\Delta\Psi_{G2}$ or $\Delta\Psi_{G100}$). MJNS without NH_4^+ is referred to throughout as the -N solution. Before applying each treatment, the -N solution was introduced to obtain a resting membrane potential, $\Delta\Psi_{-N}$, as the point of reference. Roots were allowed to equilibrate for at least 3 to 5 min in this -N solution to reach the resting potential before introducing subsequent treatment solutions.

5.2.3.1. Effect of $[NH_4^+]_o$ on $\Delta\Psi$

Roots were exposed to $[NH_4^+]_o$ of 2, 5, 10, 25, 50, 75, 100, 250, 500 μM for studying the HATS, and 1, 2.5, 5, 10, 20, 30, and 40 mM NH_4Cl for investigating the LATS, in a background of MJNS. When roots were exposed to several different $[NH_4^+]_o$ during a single impalement, G2 or G100 medium was flushed through the chamber before each change of NH_4^+ concentration. When $\Delta\Psi$ returned to its original ($\Delta\Psi_{G2}$ or $\Delta\Psi_{G100}$) value, it was satisfied that the physiological status of the root had returned to its original condition.

5.2.3.2. Effect of accompanying anion on $\Delta\Psi$

To evaluate the contribution of the accompanying anion to the observed depolarization of $\Delta\Psi$ by NH_4^+-salts in the low concentration range, $\Delta\Psi$ were measured in the following solutions in sequence: (a) 50 μM $CaCl_2$, (b) 50 μM $CaSO_4$, (c) 100 μM NH_4Cl, (d) 50 μM $(NH_4)_2SO_4$. Likewise in the high concentration range, $\Delta\Psi$ was measured in (e) 5 mM $CaCl_2$, (f) 5 mM $CaSO_4$, (g) 10 mM NH_4Cl, and then (h) 5 mM $(NH_4)_2SO_4$. These

concentrations were chosen to provide equivalent anion charge in all treatments.

5.2.3.3. Effects of metabolic inhibitors on NH_4^+-induced $\Delta\Psi$ depolarization

The same metabolic inhibitors used in the $^{13}NH_4^+$ influx study (Section 2.9.), were used to investigate effects on NH_4^+-induced depolarization of $\Delta\Psi$. These included 1 mM NaCN plus 1 mM SHAM, 10 μM CCCP, 50 μM DES, and 1 mM *p*CMBS. This study involved three steps:

(1) the responses of $\Delta\Psi$ to additions of 0.1 or 10 mM NH_4Cl were determined in sequence;

(2) the inhibitor to be evaluated was first introduced in -N solution. When $\Delta\Psi$ had reached a new steady-state, this solution was replaced with the inhibitor plus 0.1 or 10 mM NH_4Cl in sequence;

(3) the solution containing inhibitor plus NH_4Cl was replaced by -N solution.

When a new steady value of $\Delta\Psi_{-N}$ had been reached, 0.1 and then 10 mM NH_4Cl were added to the -N solution in sequence. The NH_4Cl concentrations, 0.1 or 10 mM in MJNS, were selected as representative levels for the operation of the HATS or the combined HATS and LATS (Wang et al., 1993a).

5.3. Results

5.3.1. Transmembrane electrical potentials of rice roots

Plasma membrane $\Delta\Psi$ for epidermal and cortical cells of 3-week-old rice roots (Table 11) were measured in 0.2 mM $CaSO_4$ alone ($\Delta\Psi_{CaSO4}$), or -N solution, or G2 and G100 media ($\Delta\Psi_{-N}$, $\Delta\Psi_{G2}$ and $\Delta\Psi_{G100}$, respectively). As presented in Table 11, $\Delta\Psi_{CaSO4}$ values were consistently more negative than $\Delta\Psi$ measured in other solutions. Likewise the $\Delta\Psi_{-N}$ were more negative than the corresponding $\Delta\Psi_{G2}$ or $\Delta\Psi_{G100}$ values. The depolarizing effect of NH_4Cl additions can be directly compared in Table 11 for a particular root type because -N and G2 or G100 media differed only by the presence of NH_4Cl in MJNS. Therefore, both $\Delta\Psi_{G2}$ and $\Delta\Psi_{G100}$ represented the membrane potentials of root cells adapted to their respective growth conditions.

5.3.2. Contribution of the accompany anions to $\Delta\Psi$

Figure 12 reveals that there was a very small depolarizing effect of Ca^{2+}-salts compared to NH_4^+-salts, under conditions where the concentration of the accompanying anion was held constant. Also there was virtually no difference between the depolarizing effects of Cl^- and SO_4^{2-}. This was true also at the higher concentrations of Ca^{2+}-salts and NH_4^+-salts (Traces e, f, g and h in Fig. 12). In the lower concentration range, no repolarization of $\Delta\Psi$ was observed until the Ca^{2+}-salts or NH_4^+-salts were withdrawn from the chamber. By contrast, in 5 mM $CaCl_2$, complete repolarization and even hyperpolarization was evident within 10 min of

Table 11. Membrane potentials of G2 and G100 plants measured in different bathing solutions. The bathing solution for measurements were 0.2 mM $CaSO_4$; MJNS-N; MJNS + 2 μM NH_4^+, or MJNS + 100 μM NH_4^+.

	G2 plants (mV)		G100 plants (mV)	
$\Delta\Psi_{CaSO_4}$ [a]	-140 ± 3.5	(n=5) [d]	-135 ± 1.8	(n=53)
$\Delta\Psi_{-N}$ [b]	-129 ± 1.0	(n=184)	-131 ± 0.6	(n=197)
$\Delta\Psi_{G2}$ or $\Delta\Psi_{G100}$ [c]	-116 ± 2.1	(n=14)	-89 ± 2.4	(n=28)

[a] G2 or G100 plants were impaled in 0.2 mM $CaSO_4$ solution; [b] G2 or G100 plants were impaled in -N solution; [c] G2 or G100 plants were impaled in MJNS containing either 2 μM or 100 μM NH_4Cl, respectively; [d] Average value ± standard error, n: number of observations;

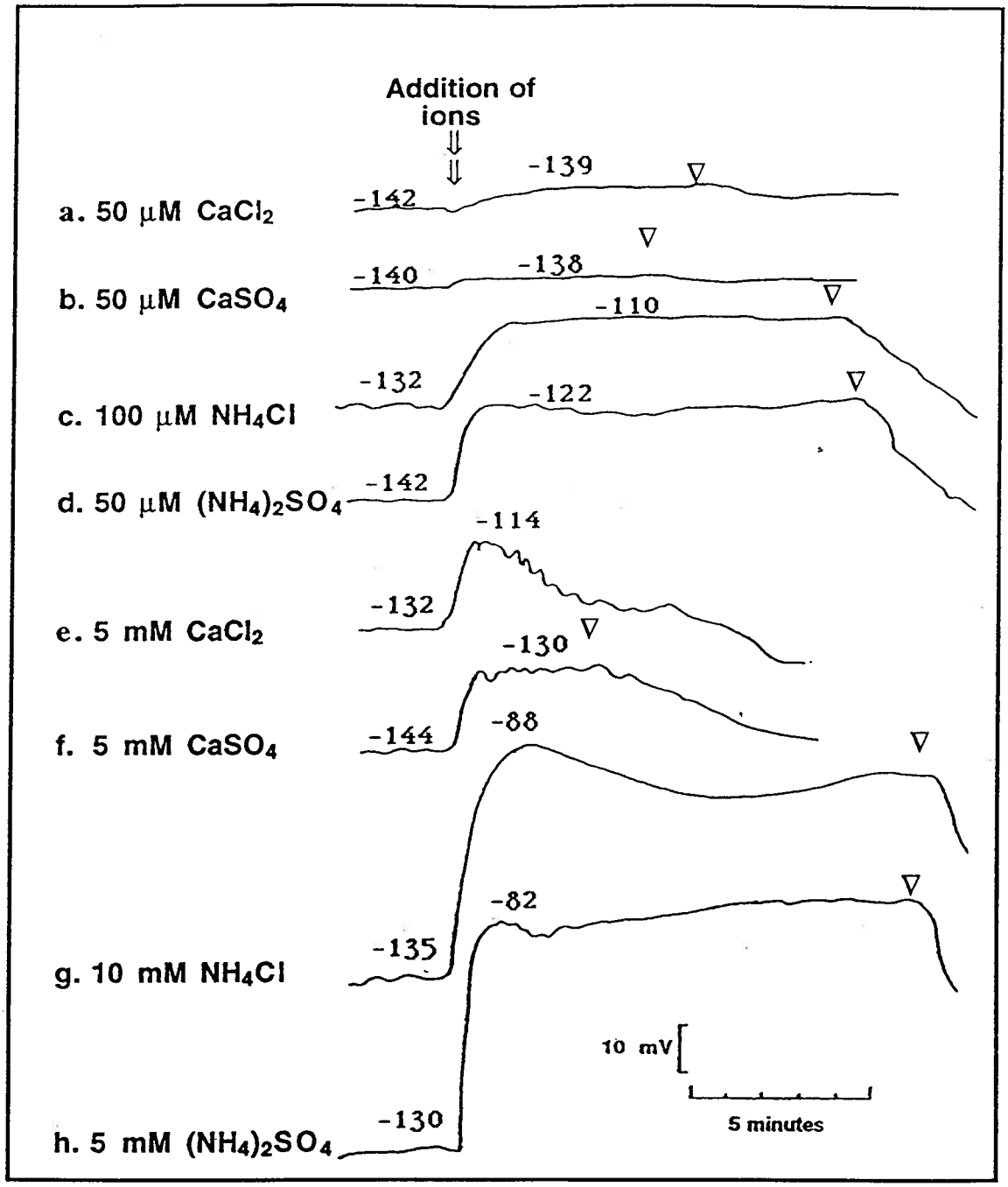


Figure 12. Effects of some anions on $\Delta\Psi$ depolarization. Representative traces to demonstrate the contribution of the accompany anions to depolarization of $\Delta\Psi$ elicited by exposure of roots to different salts at various concentrations. ∇ : the salts were withdrawn from MJNS. Each treatment was repeated on three separate plants.

evidence of repolarization in NH_4Cl (Fig. 12, trace g) but this was only partial. Only after removal of the NH_4^+-salts was complete repolarization observed.

5.3.3. Effect of $[NH_4Cl]_o$ on $\Delta\Psi$

The addition of NH_4Cl to the -N solution induced a strong depolarization of $\Delta\Psi$ (Fig. 13). This depolarization occurred rapidly after the introduction of NH_4Cl, even at very low concentrations (e.g. 2 μM NH_4Cl). The time required to reach the initial maximum depolarization was from 0.5 to 2 min, increasing with increasing $[NH_4Cl]_o$.

The depolarization of $\Delta\Psi$ was positively correlated with $[NH_4Cl]_o$. A saturable pattern was evident in the range from 2 to 1000 μM NH_4Cl (Fig. 14A) for both G2 and G100 plants. Estimated half-saturation values for net depolarization (analogous to a K_m value) were 21.8 ± 2.7 μM for G2 plants and 35.0 ± 8.0 μM for G100 plants, while the maximum depolarization (analogous to a V_{max} value) was 50.6 ± 2.0 mV for G2 plants and 34.3 ± 1.9 mV for G100 plants. Kinetic parameters were obtained by fitting the data to the Michaelis-Menten equation by means of a nonlinear regression computer program "Systat" (Wilkison, 1987) as used in our earlier kinetic study of $^{13}NH_4^+$ influx (Wang et al., 1993b). Between 1 to 40 mM $[NH_4Cl]_o$ (Fig. 14B), the magnitude of the depolarization increased linearly with increasing concentrations of NH_4Cl. This relationship was observed for both G2 and G100 rice plants, although the extent of depolarization was smaller for the latter.

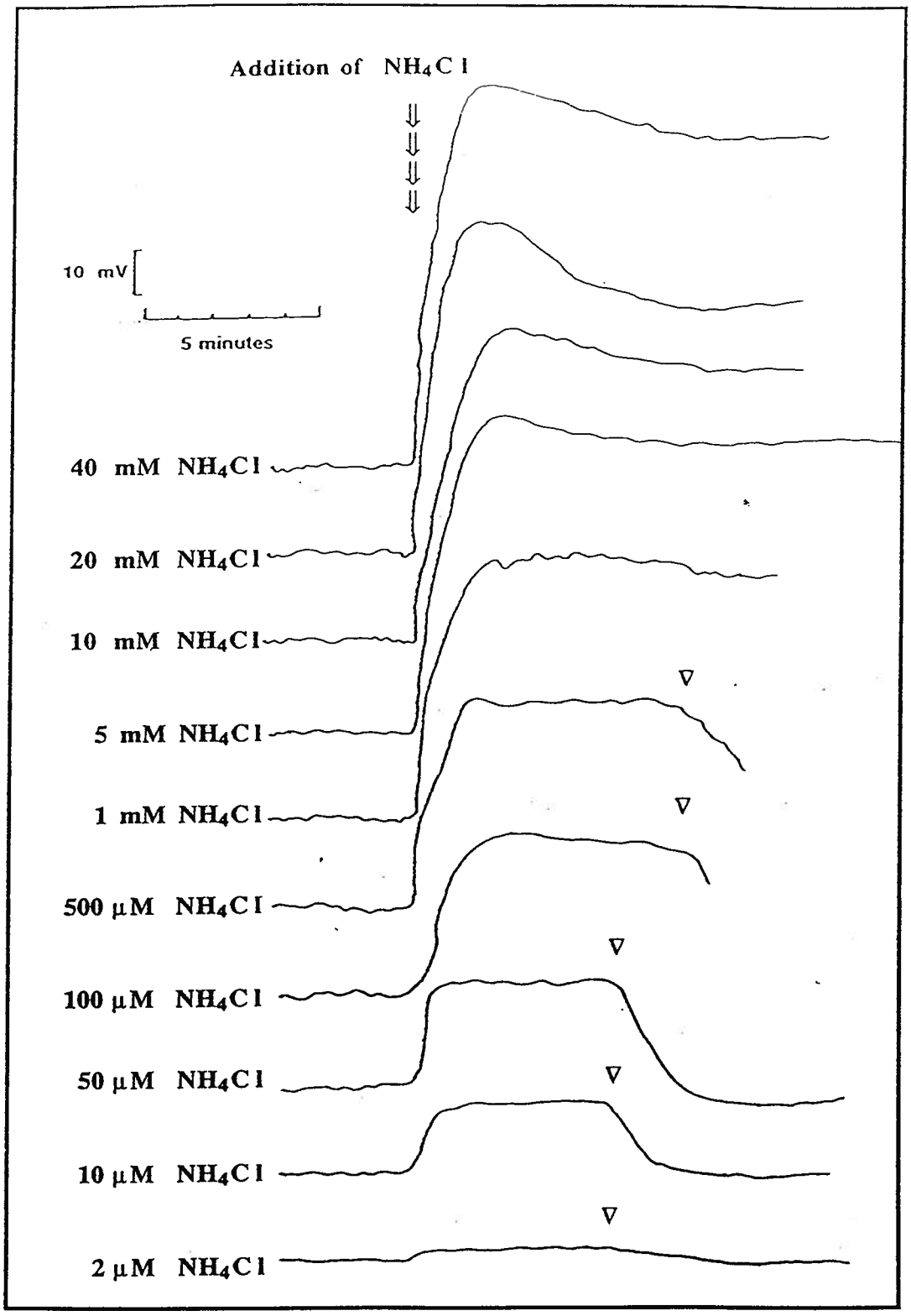


Figure 13. The $\Delta\Psi$ depolarization of root cell by NH_4Cl. Representative traces from G2 plants showing the depolarization of root cell $\Delta\Psi$ induced by adding various concentrations of NH_4Cl. ∇ : NH_4Cl was withdrawn from MJNS.

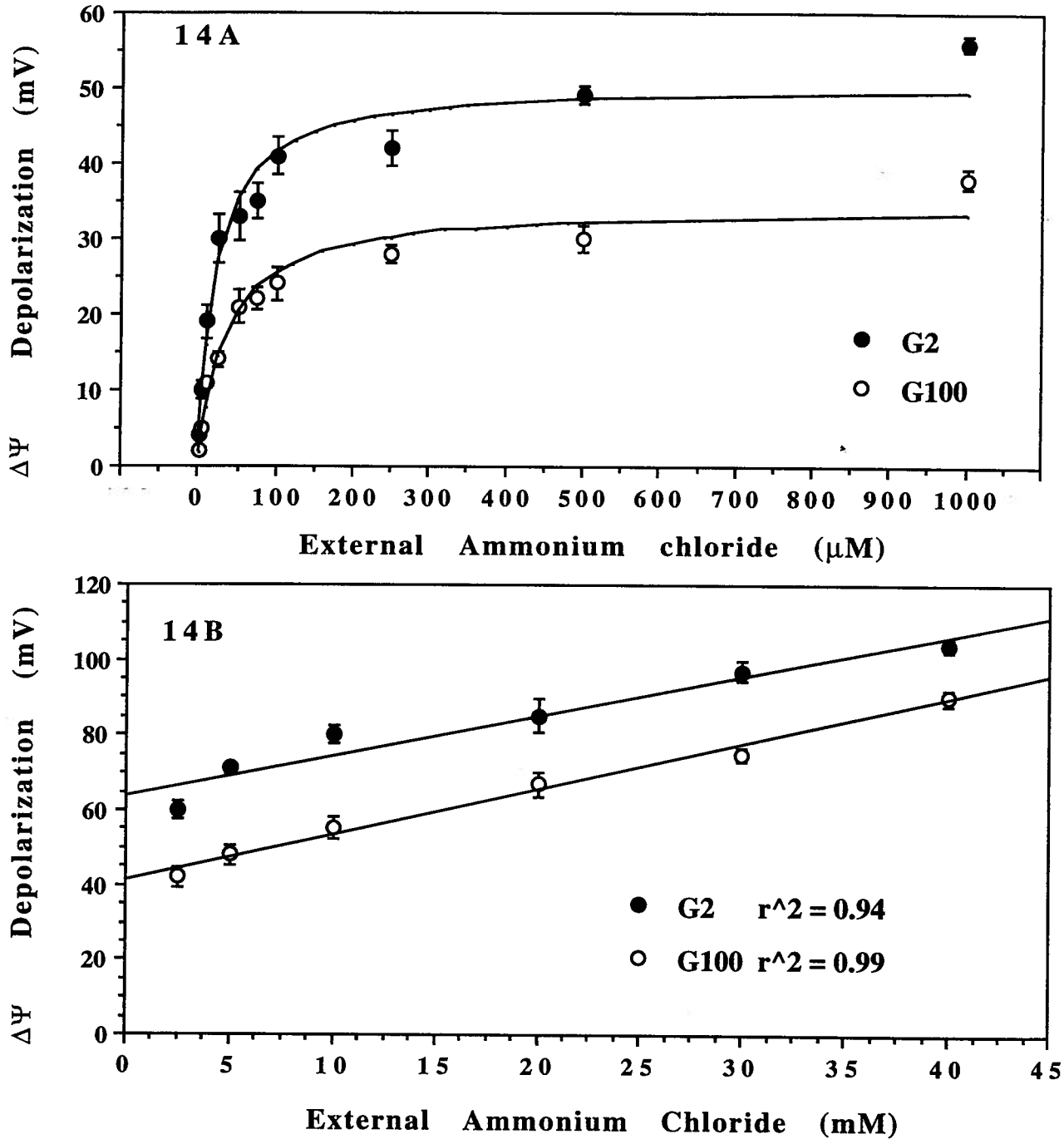


Figure 14. Concentration dependence of net ΔΨ depolarization of root cells. Rice seedlings were grown in either 100 μM (G100) or 2 μM NH_4^+ (G2). The -N media were used as basal solutions for the resting ΔΨ. Each point is the average of 3 measurements from each of 3 individual plants. The vertical bar is the standard error. 14A: Low $[NH_4Cl]_o$ range (<1 mM); 14B: High range (1 to 40 mM).

Figure 15. shows the effects of four metabolic inhibitors on $\Delta\Psi$ recorded in -N solutions. The largest depolarization of $\Delta\Psi$ (95 mV), was induced by the protonophore, CCCP, while CN^-+SHAM and the ATPase inhibitor, DES, elicited depolarizations of 82 mV and 40 mV, respectively. The external protein modifier, *p*CMBS, caused only a small depolarization (8 mV). Representative traces depicting the effects of each of these inhibitors on NH_4^+-induced depolarization of $\Delta\Psi$ are shown in Fig. 16. In Table 12, the effects of these inhibitors on the NH_4^+-induced depolarization of $\Delta\Psi$ are expressed as a percentage of the depolarization under the control conditions, in absence of the inhibitor. The data are presented as follows: (i) control: in absence of the inhibitor the reduction of NH_4^+-induced depolarization of $\Delta\Psi$ is zero; (ii) plus inhibitor: reduction of NH_4^+-induced depolarization of $\Delta\Psi$ varied from 0 to 91%, depending upon the inhibitor used and $[NH_4^+]_o$; and (iii) residual effect: the residual effect after removal of the inhibitor from external solutions on NH_4^+-induced depolarization of $\Delta\Psi$. The $[NH_4Cl]_o$ employed were 0.1 mM and 10 mM, respectively, chosen to represent the HATS and the combined HATS+LATS. In Table 12 the depolarizations of $\Delta\Psi$ caused by 0.1 mM $[NH_4Cl]_o$ were subtracted from those caused by 10 mM $[NH_4Cl]_o$ to represent the effect due to LATS alone. In the presence of the various inhibitors, the depolarization of $\Delta\Psi$ induced by HATS was generally reduced by greater than 50%. By contrast, depolarization of $\Delta\Psi$ due to NH_4^+ uptake through the LATS was only slightly affected by the presence of inhibitors. Table 12 also reveals that there was virtually no recovery from the inhibitor treatments following removal of the inhibitors from the external medium.

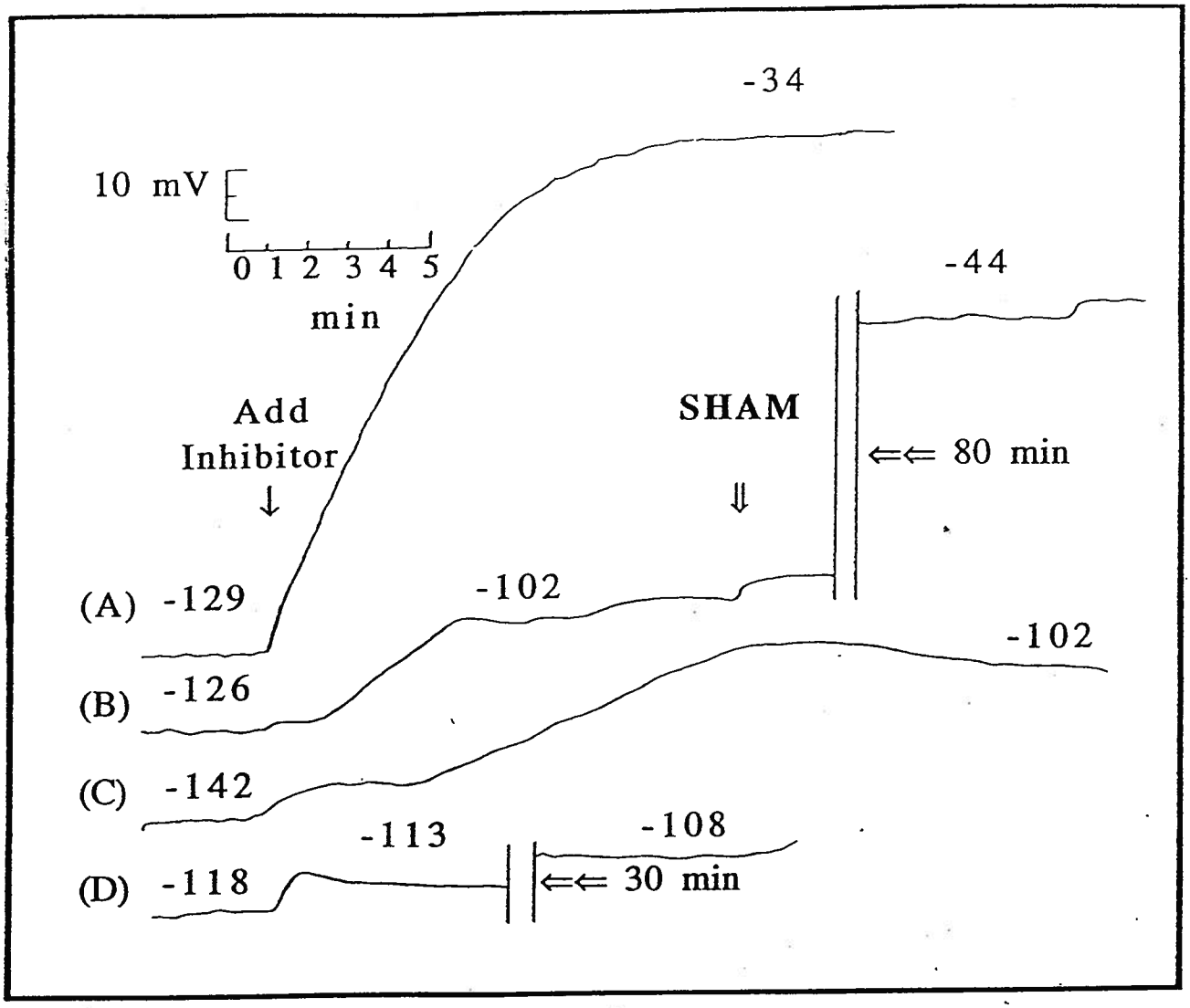


Figure 15. Effects of metabolic inhibitors on ΔΨ depolarization of root cells. Effects of metabolic inhibitors on ΔΨ depolarization of root cells. Representative traces showed effects of metabolic inhibitors on ΔΨ in time course. The inhibitors were: (A) 10 μM CCCP; (B) 1 mM CN^-+SHAM; CN^- was added into -N medium alone and then SHAM was added at (⇓); (C) 50 μM DES; (D) 1 mM *p*CMBS. Each treatment was repeated on at least three individual roots. The space between two bars (| |) is the omitted period as minutes.

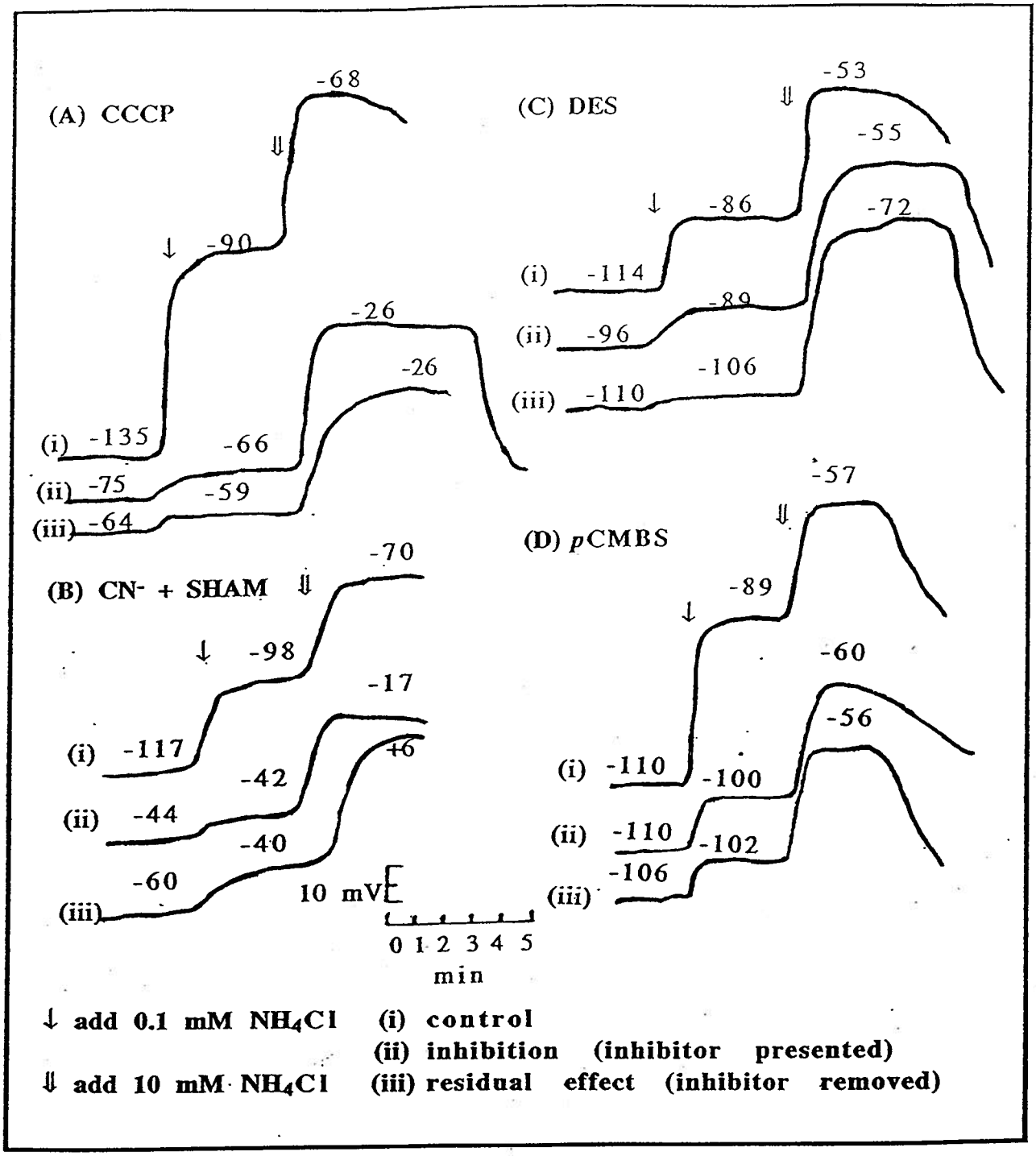


Figure 16. Effects of metabolic inhibitors on NH_4Cl induced $\Delta\Psi$ depolarization. Representative traces for the effects of NH_4Cl on $\Delta\Psi$ depolarization in the presence or absence of metabolic inhibitors in -N media. Metabolic inhibitors were those shown in Figure 15.

Table 12. Effect of metabolic inhibitors on the depolarization of $\Delta\Psi$ due to NH_4^+ uptake via HATS or LATS in G2 plants. The inhibitors used were: (A) 10 μM CCCP; (B) 1 mM CN^- + 1 mM SHAM; (C) 50 μM DES; (D) 1 mM *p*CMBS.

Inhibitor	CCCP	CN^-+SHAM	DES	*p*CMBS
Treatment	Reduction of $\Delta\Psi$ depolarization (%)			
1. Due to NH_4^+ uptake by HATS [a]				
(i) control	0	0	0	0
(ii) plus inhibitor	89	91	72	52
(iii) residual effect	91	68	81	90
2. Due to NH_4^+ uptake by LATS [b]				
(i) control	0	0	0	0
(ii) plus inhibitor	9	0	14	- [c]
(iii) residual effect	34	-	3	-

[a] The values of $\Delta\Psi$ were measured when roots were bathed in MJNS containing 0.1 mM NH_4^+ in the absence (i and iii) and presence (ii) of the inhibitors. The percentage reductions of $\Delta\Psi$ depolarization were calculated from the differences between control values for $\Delta\Psi$ induced by NH_4^+ and depolarization values in the presence of the inhibitor (ii) or after removal of the inhibitor (iii); [b] The values of $\Delta\Psi$ for LATS were the differences between measured $\Delta\Psi$ at 10 mM (for HATS+LATS) and at 0.1 mM NH_4Cl (for HATS). Then the percentage were calculated as described above (a); [c] The calculated values were negative due to the less $\Delta\Psi$ depolarization of the control.

5.4. DISCUSSION

5.4.1. Anion effect

A perennial problem associated with attempts to evaluate the electrical effect of a particular ion is the contribution of the accompanying counterion. This problem has rarely been acknowledged in published studies. However, indirect approaches, such as comparisons of the depolarizing effects of NO_3^- in NO_3^--induced and un-induced plants have been employed in order to dissect out the anion effect (Glass et al., 1992). Another approach that has proven effective is to switch from one anion to another (e.g. $CaCl_2$ to $Ca(NO_3)_2$) without changing the accompanying cation or its concentration. As a result, the observed changes of $\Delta\Psi$ are due solely to the anion effect (McClure et al., 1990; Glass et al., 1992). The results of such studies have demonstrated that NO_3^- can strongly depolarize $\Delta\Psi$ and these observations have formed the basis of currently proposed proton/nitrate cotransport mechanisms (Ullrich and Novacky, 1981; McClure et al., 1990; Glass et al., 1992).

In the present study, low concentrations of Cl^- (100 μM) provided in the form of the calcium salt elicited a very small depolarization (Fig. 12, trace a). Replacing this solution with the same concentration of $CaSO_4$ (Fig. 12, trace b) confirmed that Cl^- was responsible for most of this depolarization. Thus when these calcium salts were replaced by their ammonium equivalents, maintaining the same anion concentration, the significant depolarization of $\Delta\Psi$ could largely be attributed to NH_4^+. Although the depolarizing effects of the calcium salts, presented at 5 mM were significantly higher than at 50 μM (Fig. 12, traces e and f), the effects

of transfer to the equivalent ammonium salts can be seen to induce a much larger depolarization (53 mV compare to 18 mV; Fig. 12, traces g and e). Even though it was not possible to quantitatively isolate the contribution of Cl^- for studies of LATS, I consider that the NH_4^+ effect still predominated, even at high external $[NH_4Cl]_o$. In fact, the difference between traces g and e (Fig. 12) can be attributed to the difference between NH_4^+ and Ca^{2+} effects, since Cl^- was maintained at the same level. Thus the depolarizations referred to in the remainder of the paper were interpreted as predominantly due to the transport of NH_4^+.

A feature of these initial studies was the apparent repolarization of $\Delta\Psi$ following depolarization in the chloride solutions (Fig. 12, traces e and g) at high $[Cl^-]_o$. Although repolarization to the resting potential was not complete in 10 mM NH_4Cl, the extent of the initial repolarization was comparable to that in $CaCl_2$, where repolarization was completed. A similar spontaneous repolarization of $\Delta\Psi$ was noted in *Lemna* and in barley roots following depolarization of $\Delta\Psi$ by NO_3^- (Ullrich and Novacky, 1981; Glass et al., 1992).

5.4.2. Depolarization of $\Delta\Psi$ by HATS and LATS

Addition of ammonium chloride into -N solutions induced a rapid depolarization of membrane potential of rice epidermal and cortical cells (Figs. 12 and 13). This was evident even at very low concentration (2 μM NH_4Cl) (Fig. 13). Ullrich et al. (1984) reported that addition of NH_4^+ immediately decreased the membrane potentials of *Lemna gibba*. Likewise, the $\Delta\Psi$ of green thallus cells of *Riccia fluitans* were rapidly depolarized by $[NH_4Cl]$ as low as 1 μM (Felle, 1980). As can be seen from

Fig. 13, the time to reach initial maximum depolarization increased from ~0.5 to 3 min with increasing concentrations of NH_4Cl.

The depolarization of $\Delta\Psi$ by NH_4^+ exhibited a biphasic concentration-dependence (Figs. 14A and 14B), similar to NH_4^+ influx into roots of rice (Wang et al., 1993b). In the low concentration range (<1 mM), depolarization of the membrane potential saturated in response to $[NH_4^+]_o$ (Fig. 14A). Both net flux and unidirectional influx of NH_4^+ in rice roots have been shown to respond to $[NH_4^+]_o$ in a similar fashion (Youngdahl et al., 1982; Wang et al., 1991; 1993b). Estimated half-saturation values for NH_4^+-induced depolarization (analogous to a K_m value) were 21.8 ± 2.7 μM for G2 plants and 35.0 ± 8.0 μM for G100 plants. These values were somewhat lower than the K_m for $^{13}NH_4^+$ influx, 32 μM and 90 μM, respectively (Wang et al., 1993b). Since our studies were undertaken with the same rice variety as employed for the $^{13}NH_4^+$ influx experiments, these differences may represent differences in growth conditions for plants used for the two studies, or that membrane depolarization reflects the net, rather then the unidirectional, effect of ion fluxes. Another factor, already addressed above, is the possible effect of the accompanying anions. The maximum depolarizations (analogous to a V_{max} value) were 50.6 ± 2.0 mV and 34.3 ± 1.9 mV for G2 and G100 plants, respectively. The larger depolarizing effects of $[NH_4^+]_o$ in G2 compared to G100 plants (Figs.. 14A and 14B) correspond to the higher values of $^{13}NH_4^+$ influx observed in G2 compared to G100 plants (Wang et al., 1993b). Clearly the depolarization of $\Delta\Psi$ in response to $[NH_4^+]_o$ (<1 mM) was due to the carrier-mediated NH_4^+ uptake that exhibited Michaelis-Menten kinetics (Wang et al., 1993b). Similar saturable patterns of $\Delta\Psi$ depolarization were associated with the uptake of either NH_4^+ or NO_3^- in *Lemna* (Ullrich and Novacky, 1981; Ullrich

et al., 1984) and the uptake of both NH_4^+ and $CH_3NH_3^+$ in cells of *Riccia fluitans* (Felle, 1980).

Between 1 and 40 mM, the depolarization of $\Delta\Psi$ increased linearly with increasing $[NH_4Cl]_o$ (Fig. 14B) in a manner similar to that observed for $^{13}NH_4^+$ influx (Wang et al., 1993b). Both G2 and G100 rice plants exhibited this linear response, but the extent of depolarization was smaller in G100 plants, where $^{13}NH_4^+$ influx was also smaller. The concentration-dependent data for depolarization of $\Delta\Psi$ by LATS was fitted by linear regression with r^2 values of 0.94 and 0.99 for G2 and G100 rice plants, respectively. A similar linear response to $[NH_4^+]_o$ was reported for net NH_4^+ uptake by *Lemna* at $[NH_4^+]_o$ between 0.1 to 1 mM (Ullrich et al., 1984). However, in this concentration range, NH_4^+ uptake by *Lemna* was not associated with further depolarization of $\Delta\Psi$. Ullrich et al., (1984) interpreted this pattern as due to a diffusive uptake of NH_4^+ or NH_3. It is clear that NH_3 influx would not depolarize $\Delta\Psi$. However it is not clear how NH_4^+ uptake could occur without further $\Delta\Psi$ depolarization, unless NH_4^+ influx was associated with a stoichiometric anion influx or cation efflux resulting in an electroneutral transport.

To better understand the relationship between NH_4^+ uptake and changes in $\Delta\Psi$, the observed values of $\Delta\Psi$ depolarization were paired with the data for $^{13}NH_4^+$ influx from Wang et al. (1993b) at each $[NH_4^+]_o$ (Fig. 8). It is evident that the depolarization of $\Delta\Psi$ was strongly correlated with $^{13}NH_4^+$ influx, and that the relationship was biphasic. By use of a computer-based procedure to determine the 'break-points' for the biphasic pattern objectively (Rygiewicz et al., 1984), the correlation coefficient established a break-point at 1 mM $[NH_4^+]_o$. The biphasic pattern (Fig. 17)

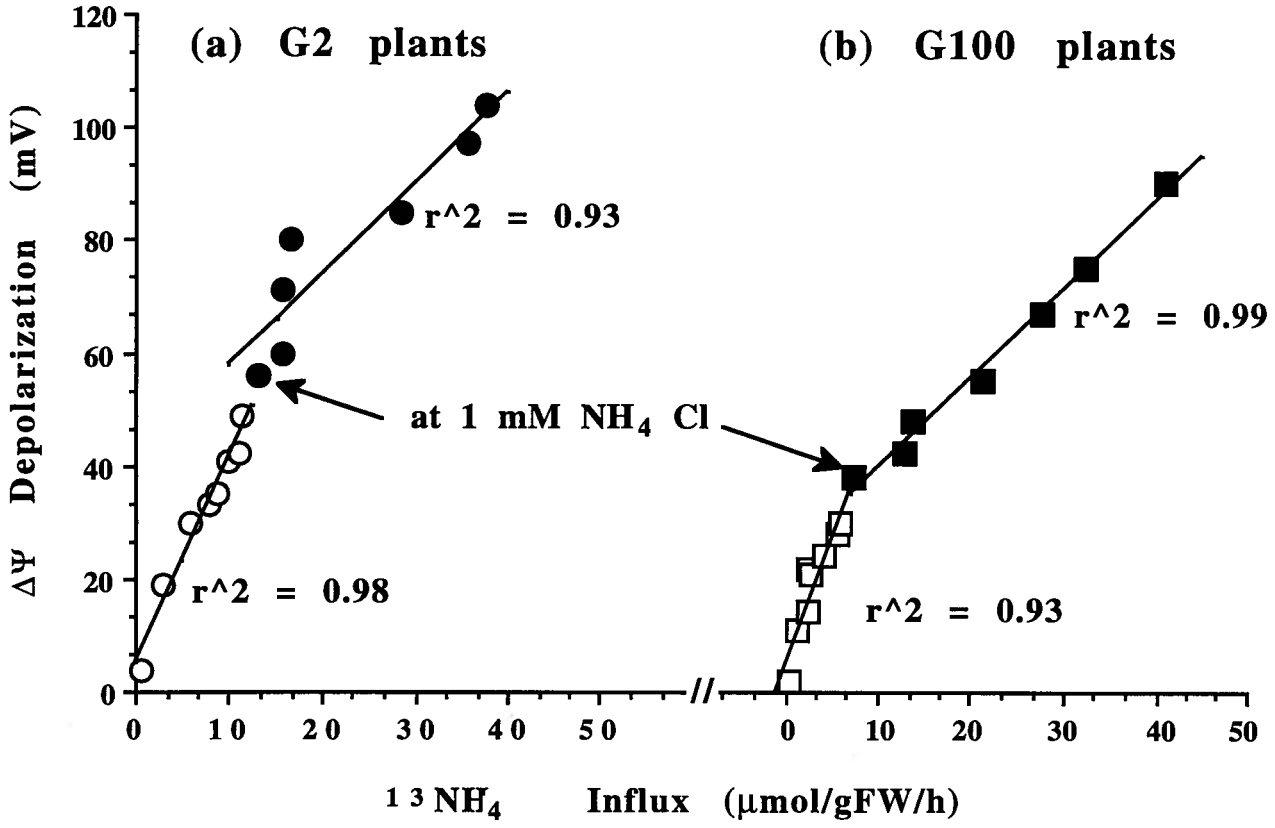


Figure 17. The relationship between $^{13}NH_4^+$ influx and $\Delta\Psi$ depolarization at the same $[NH_4^+]_o$. $^{13}NH_4^+$ influx is from Figs. 8 and 10 and net depolarization of membrane potentials is from Figs. 14 A and 14B for G2 and G100 plants measured at the same $[NH_4^+]_o$.

indicates that NH_4^+ influx and the depolarization of $\Delta\Psi$ are due to two distinct systems for NH_4^+ uptake by rice roots, i.e. a high affinity transport system (HATS) and a low affinity transport system (LATS). The larger slope of the lines for the low concentration range for G2 and G100 plants suggests that the HATS is more electrogenic than the LATS. This may be due to the increasingly electroneutral NH_4^+ transport at high $[NH_4Cl]_o$. In the present study, the electrophysiological evidence suggested that at high $[NH_4Cl]_o$ ammonium is taken up by rice roots in the cation form (NH_4^+) despite the presence of a relatively high concentration of NH_3 in solution. Alternatively, it might be argued that depolarization of $\Delta\Psi$ may be due to the inhibition of the H^+-ATPase by NH_3 at high $[NH_4^+]_o$. However, the lack of a pronounced increase of ^{13}N uptake at pH values approaching the pKa for NH_4^+ does not support this interpretation (Wang et al., 1993b). In addition, the rapid repolarization of $\Delta\Psi$ following removal of external NH_4^+ (in Fig. 12, traces g and h) is unexpected considering that the $t_{1/2}$ for cytoplasmic ^{13}N exchange is ~7 min (Wang et al., 1993a).

5.4.3. Calculation of the free energy for NH_4^+ transport

The average $\Delta\Psi$ values were substantially more negative in G2 plants impaled in 2 μM NH_4^+ than in G100 plants impaled in 100 μM NH_4^+ (Table 11). Furthermore, the extent of the depolarization of $\Delta\Psi$ by NH_4^+ was consistently greater for G2 plants than G100 plants at a particular $[NH_4^+]_o$. The average $\Delta\Psi$ value was -116 mV for G2 plants and -89 mV for G100 plants (Table 11). For both G2 and G100 plants, the resting potentials in the absence of NH_4^+ ($\Delta\Psi_{-N}$) were in the range of -120 to -140 mV. In low salt bathing medium (0.2 mM $CaSO_4$), the transmembrane electrical potentials ($\Delta\Psi_{0.2\ mM\ CaSO_4}$) were 25 mV more negative than $\Delta\Psi_{-N}$ and 45 mV

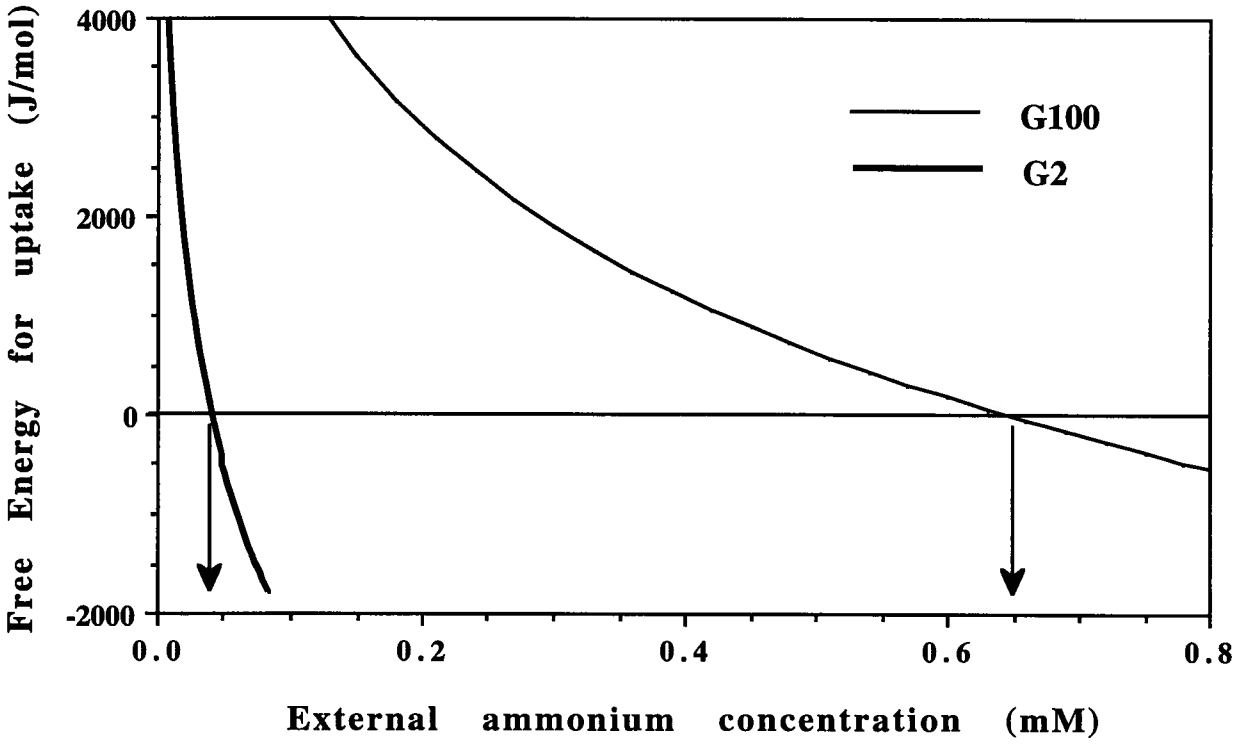


Figure 18. Free energy requirement for NH_4^+ uptake as a function of external $[NH_4^+]$. Values of cytoplasmic $[NH_4^+]$ were taken from our previous study (Wang et al., 1993a). Arrows indicate the $[NH_4^+]_o$ below which NH_4^+ uptake is against the electrochemical potential gradient for G2 and G100 plants, respectively.

more negative than $\Delta\Psi_{G2}$ and $\Delta\Psi_{G100}$, respectively. These differences reflect the contributions to the membrane depolarization from the various ions present in MJNS. Since the values of $\Delta\Psi_{-N}$, and $\Delta\Psi_{G2}$ and $\Delta\Psi_{G100}$, were measured in the same basal medium (MJNS), the observed differences must largely be due to the $[NH_4^+]_o$ in the bathing medium.

The measured $\Delta\Psi$, together with values for cytoplasmic $[NH_4^+]$, are needed to estimate the electrochemical potential difference for NH_4^+ across the plasma membrane, which in turn allows us to determine the energy requirement for transport (Findlay and Hope, 1976). Taking 3.72 mM and 20.55 mM as cytoplasmic $[NH_4^+]$, and -116 mV and -89 mV as steady-state $\Delta\Psi$ for G2 and G100 roots, respectively (Wang et al., 1993a), at a series of given $[NH_4^+]_o$ the Nernst potentials (E_N) were estimated for G2 and G100 roots, respectively. From these values, the free energy ($\Delta\mu_{io}$) required to transport NH_4^+ across the plasma membrane can be computed from the differences between measured membrane potentials ($\Delta\Psi_{G2}$ or $\Delta\Psi_{G100}$) and estimated Nernst potentials at specific $[NH_4^+]_o$ (Fig. 18). The estimated free energy differences ($\Delta\mu_{io}$) for NH_4^+ distribution were positive at or below 42 μM for G2 and 655 μM for G100 plants (Fig. 18). This means that below these concentrations, NH_4^+ uptake by G2 and G100 roots respectively, must be active (Fig. 18). These concentrations represent the lower limits for active transport under steady-state conditions. However, displacing $[NH_4^+]_o$ to values greater than 2 or 100 μM, respectively, will elevate the limit for active transport because of further $\Delta\Psi$ depolarization and increased cytoplasmic $[NH_4^+]$. Above these minimum levels, the uptake of NH_4^+ may occur via passive transport systems, down the electrochemical potential gradients for NH_4^+. As pointed out previously (Wang et al., 1993b), these free energy estimations only provide a prediction of the feasibility of the uptake process occurring under the prescribed conditions. For both G2 and

G100 plants, the predicted $[NH_4^+]_o$ for the shift from active to passive uptake was quite a bit lower than the break-point determined by the kinetics analyses (42 μM and 655 μM versus 1 mM). Thus, one must be cautious in identifying a specific transport system based purely on thermodynamic or kinetic considerations.

5.4.4. Mechanisms of NH_4^+ uptake by HATS and LATS

The preceding section has demonstrated that at low $[NH_4^+]_o$ (< 42 μM for G2 plants and 655 μM for G100 plants), NH_4^+ influx appears to be an active process in roots of rice plants. However, the details of this mechanism are unknown for rice and for any higher plants. Possible mechanisms for this active uptake via HATS include: (a). a proton : NH_4^+ symport; (b). a specific NH_4^+ ATPase. The results of the inhibitor studies, both for the electrical potentials in the present study and $^{13}NH_4^+$ influx (Wang et al., 1993b) provide evidence for a dependence (either direct or indirect) on the proton motive force. Application of CCCP caused 89% and 85% inhibition, respectively, of membrane depolarization by NH_4^+ and $^{13}NH_4^+$ influx in solution containing 100 μM NH_4^+. The strong inhibitory effects of CN^-+SHAM on depolarization of $\Delta\Psi$ (91%) and on $^{13}NH_4^+$ influx (81%) confirm the dependence of these processes on a source of metabolic energy without distinguishing the nature of the mechanisms. The effects of DES, an inhibitor of the H^+-ATPase, indicated the involvement of the proton pump, suggesting speculatively that H^+-transport might be involved.

The results of the present and earlier studies (Wang et al., 1993b), strongly suggest that the two systems, HATS and LATS, have different mechanisms of energy coupling. Above 42 μM for G2 and 655 μM for G100

plants, NH_4^+ transport was predicted to be a passive process. This prediction is borne out by the generally smaller effects of metabolic inhibitors at high external $[NH_4^+]$ than at low $[NH_4^+]_o$ (present study and in Wang et al., 1993b), although $^{13}NH_4^+$ influx showed greater sensitivity to inhibitors than the $\Delta\Psi$ depolarization. There is virtually no information available regarding the energy coupling for the LATS. Passive entry of NH_4^+ might occur via an electrogenic uniport (Kleiner, 1981; Ullrich et al., 1984). This may be a specific channel for NH_4^+ or a shared cation channel. For example, the recently described K^+ channel in *Arabidopsis* has been shown to have an NH_4^+ conductance that is ~30% of the K^+ conductance (Schachtman et al., 1992). Also, in the cyanobacterium *Anabaena variabilis* (Avery et al., 1992), the uptake of Cs^+ (a K^+ analog at the uptake step) and NH_4^+ was closely related. Thus low affinity NH_4^+ transport might occur via the K^+ channel.

5.5. SUMMARY

The transmembrane electrical potential differences ($\Delta\Psi$) were measured in epidermal and cortical cells of intact roots of 3-week-old rice (*Oryza sativa* L. cv. M202) seedlings grown in 2 or 100 micromolar (μM) NH_4^+ (G2 or G100 plants, respectively). In modified Johnson's nutrient solution (MJNS) containing no nitrogen, $\Delta\Psi$ was in the range of -120 to -140 millivolts (mV). Introducing NH_4^+ to the bathing medium caused a rapid depolarization. At the steady-state, average $\Delta\Psi$ of G2 and G100 plants were -116 mV and -89 mV, respectively. This depolarization exhibited a biphasic response to external $[NH_4^+]$ similar to that reported for $^{13}NH_4^+$ influx isotherms (Wang et al., 1993b). Plots of membrane depolarization versus $^{13}NH_4^+$ influx were also biphasic, indicating distinct

coupling processes for the two transport systems, with a break-point between two concentration ranges around 1 mM NH_4^+. The extent of depolarization was also influenced by nitrogen status, being larger for G2 plants than G100 plants, corresponding to the larger NH_4^+ influxes in G2 plants than G100 plants. Depolarization of $\Delta\Psi$ due to NH_4^+ uptake was eliminated by a protonophore (carboxylcyanide-*m*-chlorophenyl-hydrazone), inhibitors of ATP synthesis (sodium cyanide plus salicylhydroxamic acid), or an ATPase inhibitor (diethylstilbestrol).

Chapter 6. REGULATION OF AMMONIUM UPTAKE

6.1. INTRODUCTION

When plants are deficient in nutrients, such as PO_4^{3-}, SO_4^{2-}, Cl^-, their uptake capacity is greatly enhanced (Lee, 1982). This phenomenon has been known since the works of Brezeale (1907 in Glass, 1989) that nutritional history of a plant can profoundly affect its subsequent capacity to absorb the same ion (see also Hoagland and Broyer, 1936; Broyer and Hoagland, 1943). Such relationships between the ions provided during plant growth and their subsequent uptake by roots or tissues was well defined in several species for the uptake of K^+ (Leigh and Wyn Jones, 1973; Glass, 1975; 1976; 1978; Pettersson, 1975; Dunlop et al., 1979; Jensen and Pettersson, 1979; Pettersson and Jensen, 1979), Cl^- (Sanders, 1980; Smith and MacRobbie, 1981; Greenway, 1965; Pitman, 1971; Cram, 1973; Hodges and Vaadia, 1964), PO_4^{3-} (Lefebvre and Glass, 1982; Lee, 1982) SO_4^{2-} (Lee, 1982) and NO_3^- (Jackson et al., 1974; MacKown et al., 1982; Glass et al., 1985; Siddiqi et al., 1989, 1990; Jackson and Volk, 1992; King et al., 1993). However, the quantitative basis of the correlation between the rate of N absorption and the N-status of the plant material is not precise (Lee and Rudge, 1986).

It have been demonstrated that plants are able to adapt to available sources of N over a wide range of concentrations (Clement et al., 1978; Wang et al., 1991). The existence of distinct transporters with different affinities for either nitrate or ammonium (Siddiqi et al., 1989; Wang et al., 1993b) represents an important part of this capacity for adaptation. Typically, nitrogen starvation leads to elevated fluxes of nitrogen, while N

excess leads to down regulation of uptake. However, the underlying mechanisms responsible for these changes are largely unknown. Several hypotheses have been advanced concerning the sources of feedback regulation responsible for controlling N uptake. These include the importance of products of N assimilation (Lee and Rudge, 1986; Cooper and Clarkson, 1989; Jackson and Volk, 1992), as well as the effects of accumulated ions (NO_3^- and NH_4^+) on influx or efflux (Morgan and Jackson, 1988a, 1988b; Siddiqi et al., 1989; King et al., 1993; Wang et al., 1993a).

It has been suggested by Morgan and Jackson (1988b), that at high plant N status, reduction or suppression of net ammonium uptake may be due to (i) low energy supply to the root system, (ii) accumulation in the root tissue of a nitrogenous compound which exerts negative feedback on the influx system, (iii) high efflux of endogenous NH_4^+. This accumulated regulating effector could be ammonium ions generated by degradation of organic nitrogenous sources within roots, or rapid accumulation of ammonium in N-depleted roots upon initial exposure to ammonium, or relative ease of outward ammonium movement (Morgan and Jackson, 1988a, 1988b). The regulation of influx may therefore reflect the interplay among suppression of influx by a product of ammonium assimilation, the accumulation of root ammonium and associated ammonium efflux, and a stimulation by ammonium of its own uptake (Morgan and Jackson, 1992).

It was found that $^{13}NH_4^+$ influxes into intact roots of rice were negatively correlated with the level of NH_4^+ provision during growth and the internal [NH_4^+] in root tissues (Wang et al., 1993a, 1993b). It has been suggested that the regulation of NH_4^+ uptake could result from feedback effects of accumulated NH_4^+ or products of NH_4^+ assimilation (Ullrich et al., 1984; Lee and Rudge, 1986; Morgan and Jackson, 1988; Lee et al., 1992;

Jackson and Volk, 1992; Wang et al., 1993a). These exert effects on both influx and efflux although the principle effect is upon influx (Wang et al., 1993a). However, the mechanism(s) of regulation are still unclear.

In order to explore the basis of the negative feedback regulation of NH_4^+ uptake, I investigated the effects of the following pretreatments on $^{13}NH_4^+$ influx: (1) repleting N-depleted plants in 1 mM NH_4^+ in the presence or absence of MSX; (2) depleting N-repleted plants in 2 μM NH_4^+ solution in the presence or absence of MSX; (3) elevating root glutamine concentrations by supplying this amino acid exogenously; (4) altering internal concentrations of NH_4^+, glutamine and other amino acids in root tissue of the above treatments; (5) using selected inhibitors of ammonium assimilation to study the effect of perturbing ammonium metabolism on ammonium uptake. The results of these experiments are interpreted in terms of a cascade model for the regulation of NH_4^+ influx in rice roots.

6.2. MATERIALS AND METHODS

6.2.1. Plant growth and ^{13}N production

Section 2.2. Seed germination; Section 2.3. Growth conditions; Section 2.4. Provision of nutrients; Section 2.5. Production of $^{13}NH_4^+$.

6.2.2. Experimental design

6.2.2.1. Experiment I. Depletion and repletion study

To investigate NH_4^+ uptake by roots in response to changing plant N status, $^{13}NH_4^+$ influx was measured in NH_4^+-repleted G2 plants or NH_4^+-depleted G1000 plants as well as G2 and G1000 plants under their growth conditions. At designated times, the assigned G2 plants were transferred to the G1000 medium and G1000 plants were transferred to the G2 medium. The time periods of repletion were 1, 2, 3, 3.5, 4, 4.5, 5, 6.5, 7.5, 8, 9.5, 12, 13.5, 24, 48, 72 h. The time periods of depletion were 0, 0.33, 0.58, 0.92, 1.75, 2.75, 3.75, 12, 18, 25, 50, 60, 72, 97, 126, 145, 161, 192 h.

6.2.2.2. Experiment II. Effects of MSX

The objective of this study was to investigate the time course of effects of MSX on $^{13}NH_4^+$ influx. Either G2 or G1000 plants were pretreated in their respective growth media in the presence of 1 mM MSX (G2+MSX or G1000+MSX) for 1, 4, 12 and 24 h before the $^{13}NH_4^+$ influx measurement. A second set of plants was used to investigate MSX effects during repletion and depletion: plants were first transferred into growth media with MSX containing the same $[NH_4^+]_o$ as they had been grown in (i.e. in G2+MSX for G2 plants or G1000+MSX for G1000 plants) at 24 h before measurement, and then G2 plants were transferred from G2+MSX to G1000+MSX or G1000 plants were transferred from G1000+MSX to G2+MSX at times of 1, 4, 12 and 24 h. For comparison, a third set of plants was transferred from growth medium to pretreatment medium i.e. G2 plants to G1000 medium or G1000 plants to G2 medium at times of 1, 4, 12 and 24 h. In another experiment, the pretreatment times for both G2 plants repleted in G1000

medium and G1000 plants depleted in G2 medium were 0, 1, 4, 12, and 24 h. The influxes were measured for 10 min in 100 μM $^{13}NH_4^+$-labeled solution without MSX. Each datum point is the mean of 6 replicates and the vertical bar represents the standard error (± se).

6.2.2.3. Experiment III. Effects of exogenous amino acids

(1) Effects of pretreatment with glutamine on $^{13}NH_4^+$ influx of rice roots: G100 plants were pretreated in G100 medium with or without 10 mM glutamine for 16 h before measuring $^{13}NH_4^+$ influx. $^{13}NH_4^+$ influxes were then measured in 2, 10, 25 and 100 μM $^{13}NH_4^+$-labeled solution without glutamine. (2) The effects of various exogenously supplied amino acids on the influx of $^{13}NH_4^+$: G2 plants were pretreated in G2 medium or G100 medium plus 10 mM glutamate, glutamine or asparagine for 16 h, respectively. $^{13}NH_4^+$ influxes were measured in 100 mM labeled $^{13}NH_4^+$ solution in the presence of the same amino acids. Each experiment was repeated twice, with 3 replicates.

6.2.2.4. Experiment IV. Effects of selected inhibitors

Inhibitors of glutamine synthesis (L-methionine DL-sulfoximine, MSX), glutamate synthesis (6-diazo-5-oxo-L-norleucine, DON) and aminotransferases (amino-oxyacetate, AOA) were used to perturb tissue concentrations of glutamine and glutamate to investigate the effect of change of these compounds on $^{13}NH_4^+$ influx. All treatments of inhibitors were administered for 16 h at 100 mM. $^{13}NH_4^+$ influxes were measured in either 100 mM or 10 mM labeled $^{13}NH_4^+$ solution.

6.2.3. Determination of free ammonium in root tissue

See section 2.5.

6.2.4. Determination of amino acids in root tissue

See section 2.8.

6.3. RESULTS

6.3.1. Experiment I. Depletion and repletion study

As shown in Fig. 19, the initial $^{13}NH_4^+$ influx of nitrogen-deficient rice plants (G2 plants) was 11.10 μmol $g^{-1}FW\ h^{-1}$, which is close to the V_{max} (12.8 μmol $g^{-1}FW\ h^{-1}$) of G2 plants (Wang et al., 1993b). After repletion in G1000 medium, influx increased to nearly 3 times its initial value (to 31.97 μmol $g^{-1}FW\ h^{-1}$) during the first 5 h. Between 6 to 12 h of loading, influxes declined to about 10 μmol $g^{-1}FW\ h^{-1}$. After three days in 1 mM NH_4^+ solution, the $^{13}NH_4^+$ influx dropped below 5 μmol $g^{-1}FW\ h^{-1}$. When G2 plants were repleted in 10 or 100 μM NH_4^+ solution, G2 roots responded with a similar pattern, but showed a delay in reaching the maximum of influx (data not shown).

Nitrogen-sufficient rice seedlings were grown in G1000 medium for at least 13 days and transferred to G2 medium for periods varying from 0.3 to 192 h, respectively, before measurement of $^{13}NH_4^+$ influx. As shown in Fig. 20A, initial $^{13}NH_4^+$ influx of G1000 plants was quite low (1.15 μmol $g^{-1}FW\ h^{-1}$) in agreement with previous reports (Wang et al., 1993b). Short-term depletion in G2 medium, for periods of 0.5 to 4 h, caused $^{13}NH_4^+$ influxes to increase almost 10 fold. Between 4 to 24 hours, $^{13}NH_4^+$ influx of these N-depleted plants was close to the V_{max} for $^{13}NH_4^+$ influx of G2

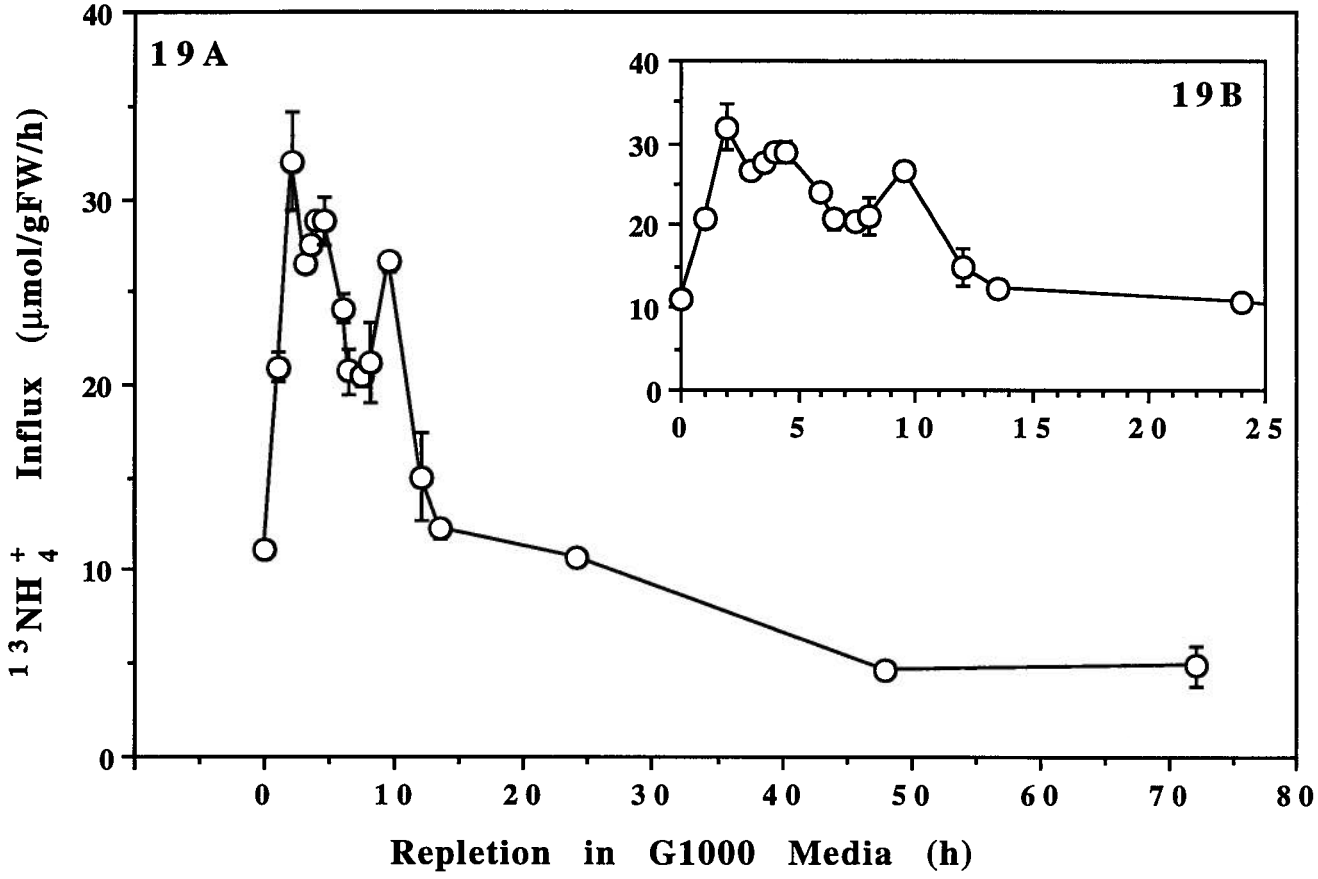


Figure 19. $^{13}NH_4^+$ influx of repleted G2 plants. After repletion in G1000 medium for various periods, $^{13}NH_4^+$ influx of G2 plants was measured in 100 µM $^{13}NH_4^+$-labeled solutions. Insert 19B shows, in expanded form, the first 24 h of repletion. Each datum point is the mean of 3 to 6 replicates and the vertical bar represents the standard error (± se).

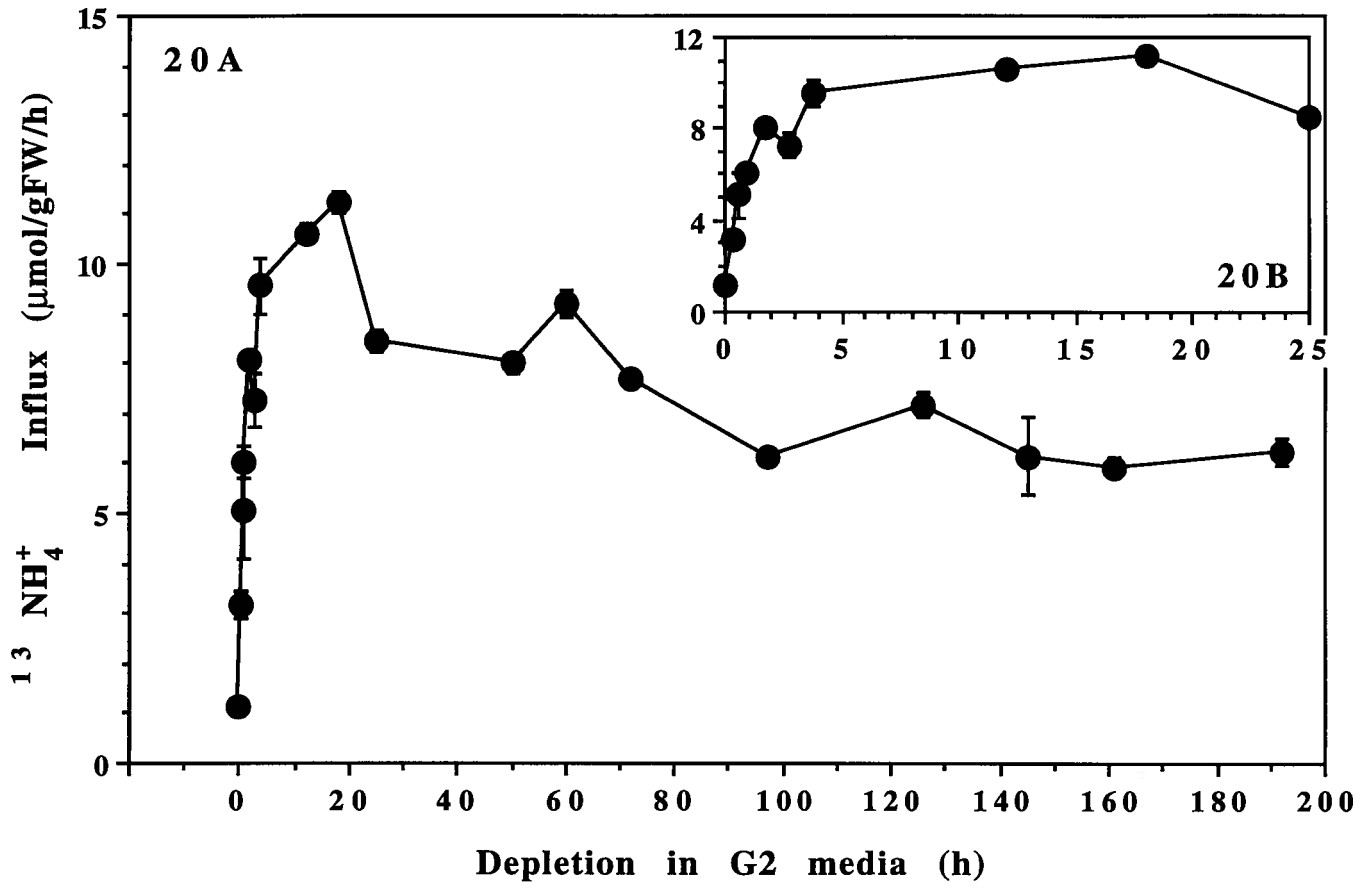


Figure 20. $^{13}NH_4^+$ influx of G1000 plants during depletion in G2 medium for various periods. The influxes were measured in 100 µM $^{13}NH_4^+$-labeled solution. Insert 20B shows in expanded form the data for the first 24 h of depletion. Each datum point is the mean of 3 to 6 replicates and the vertical bar represents the standard error (± se).

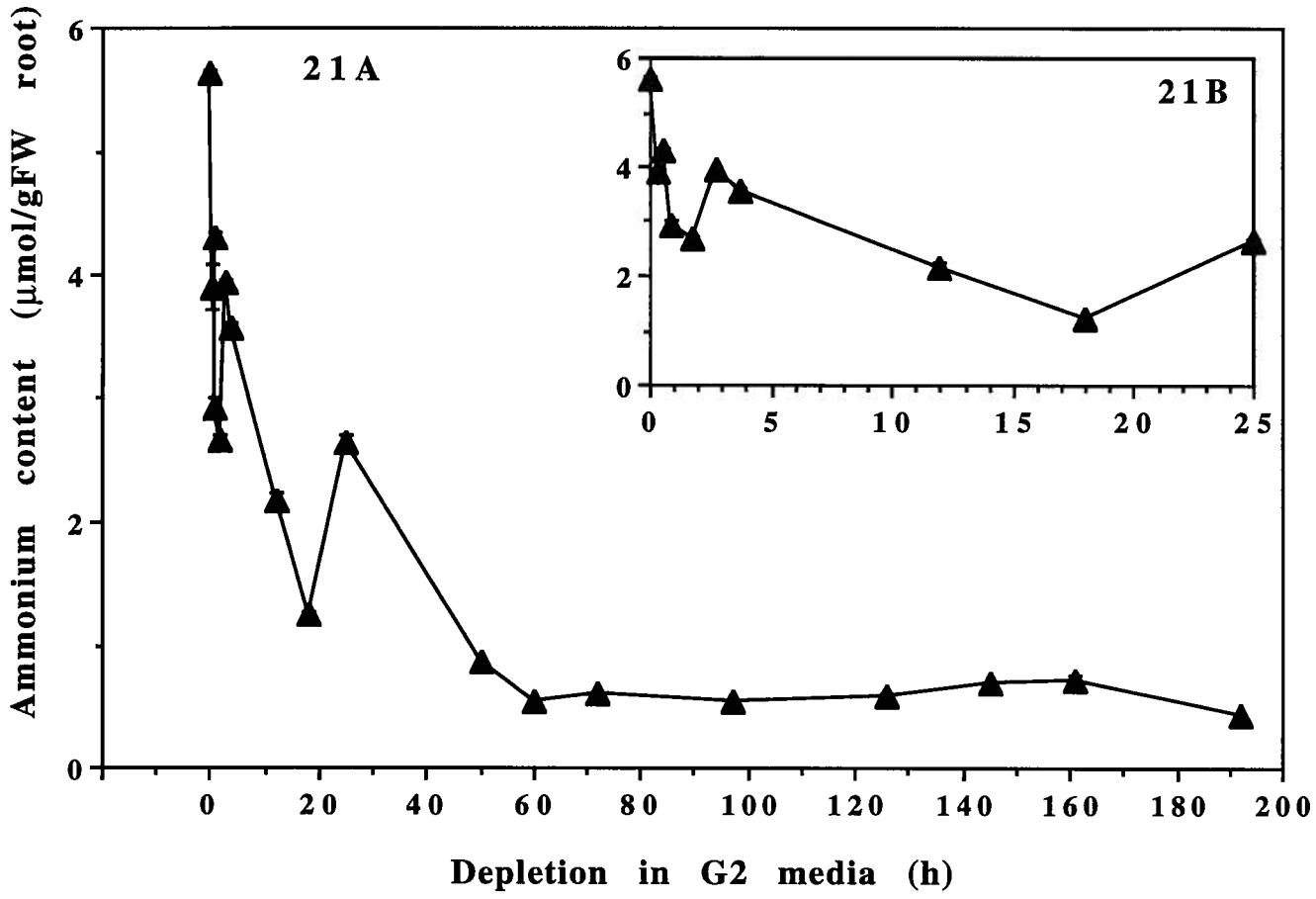


Figure 21. Internal ammonium content of depleted G1000 roots. G1000 roots were depleted in G2 medium for various periods and internal ammonium content were assayed. Insert 21B shows in expanded form the data for the first 24 h of depletion. Each datum point is the mean of 6 replicates and the vertical bar represents the standard error (± se).

plants (~11 μmol $g^{-1}FW\ h^{-1}$) (Fig. 20B). After 24 hours depletion, the $^{13}NH_4^+$ influxes declined but were still higher than those of G1000 plants at steady-state. The results indicated, that depletion in G2 medium for up to 8 days, caused no further decline of influx, which remained at about 6 μmol $g^{-1}FW\ h^{-1}$. Meanwhile, root NH_4^+ concentrations dropped rapidly during the first 4 h depletion of N, from 5.6 to 3.6 μmol $g^{-1}FW$ (Fig. 21A). After 24 h depletion, internal $[NH_4^+]$ remained at a low level (~0.6 μmol g^{-1} FW, in Fig. 21B). Figures 20B and 21B reveal that there was a negative correlation ($r^2 = 0.74$) between $[NH_4^+]_i$ and $^{13}NH_4^+$ influx during 24 h depletion of N. Beyond 24 h of N depletion, no correlation was found. Changes of the total AA content in root tissue of G1000 plants during depletion in G2 medium, are presented in Fig. 22A. In the first 4-5 h of depletion of N, total amino acid concentration ($[AA]_i$) increased (Fig. 22B). In fact the total $[AA]_i$ remained above the original level through 200 h of depletion. The contents of the major amino acids and amides, $[Gln]_i$, $[Glu]_i$, $[Asn]_i$, and $[Asp]_i$ were also found to have increased in the same fashion (data not shown).

The phenomenon of stimulated influx observed during the first hours following exposure of G2 plants to 1000 μM NH_4^+ was not as pronounced in the second experiments (open circles in Fig. 23A) as in the first experiment (Fig. 19A). This may have been due to differences of experimental conditions. In the first experiment, the depletion/repletion was carried out in a large volume of nutrient solution (in 35-liters Plexiglas tanks) in which the NH_4^+ concentrations were held relatively constant. In the second experiment, the same treatments were performed in a volume of 20 ml of medium. Such a small volume may have limited the repletion process and consequently affected the extent of the influx response. For example, typical cytoplasmic and vacuolar $[NH_4^+]$ were 0.19 and 2.19 μmol $g^{-1}FW$ for

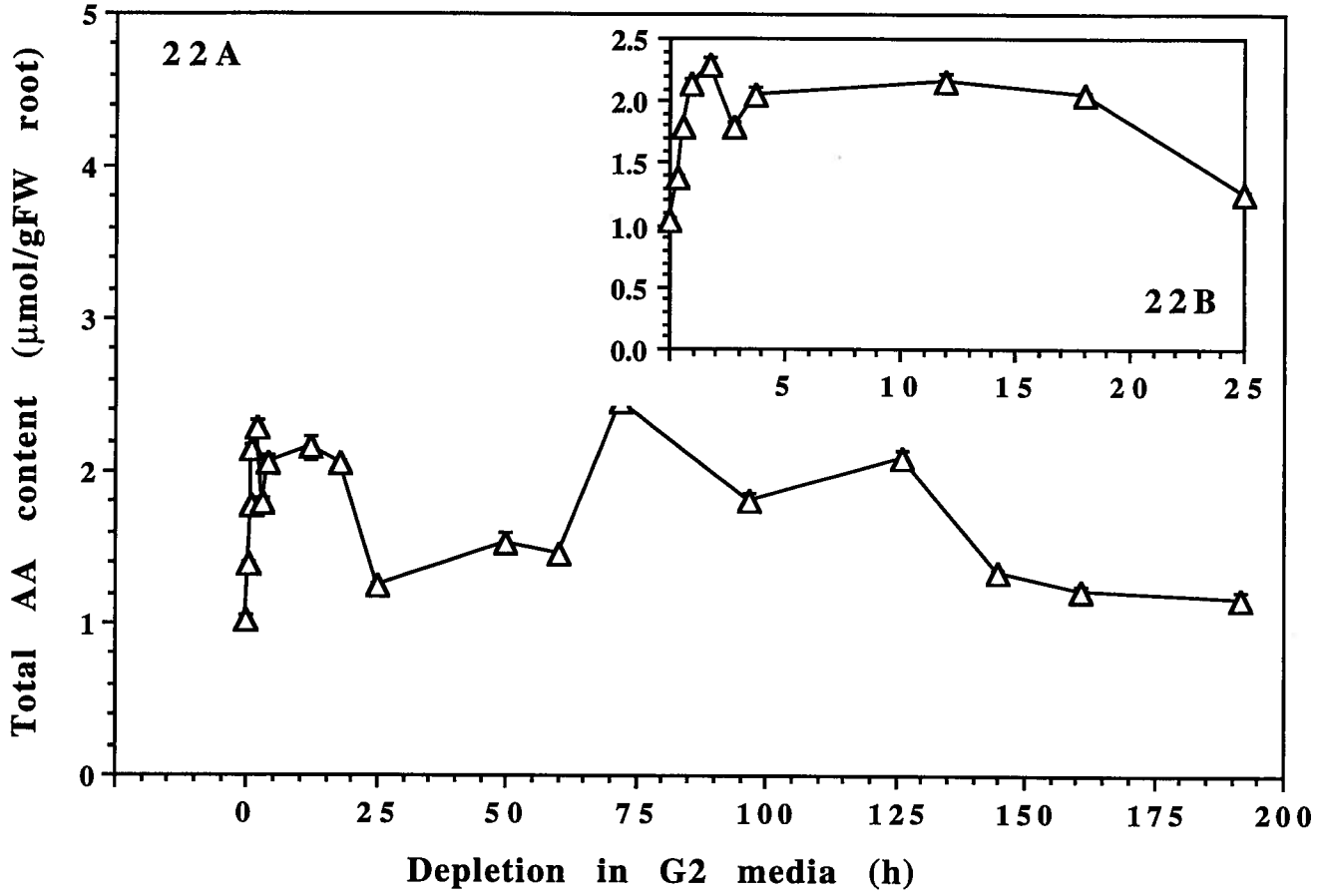


Figure 22. Total amino acid concentration ($[AA]_i$) of depleted G1000 roots. After depletion in G2 medium for various periods, G1000 roots were assayed for tissue amino acid concentration ($[AA]_i$). Insert 22B shows in expanded form the data for 24 h of repletion. Each datum point is the mean of 6 replicates and the vertical bar represents the standard error (± se).

G2 roots and 1.94 and 4.91 μmol g^{-1}FW for G1000 roots, respectively (see Table 4). This means that in order to convert G1000 plants to G2 plants there is about 4.47 μmol NH_4^+ g^{-1}FW to be depleted either by metabolism or efflux to the external media. Assuming that rates of efflux and assimilation are equivalent at about 20% of the rate of influx (Chapter 3 and Wang et al., 1993a), then the release of NH_4^+ could elevate external [NH_4^+] to nearly 100 μM. In the small volume employed for this experiment the released NH_4^+ would readily be re-absorbed, slowing down the change from G1000 to G2 statues.

As shown in Fig. 23A, when G2 plants were repleted with NH_4^+ in G1000 medium, the $^{13}NH_4^+$ influx (closed circle) increased from 8.17 to 10.00 μmol g^{-1}FW h^{-1} during the first hour, then dropped to 8.61 at 4 h and 1.95 μmol g^{-1}FW h^{-1} after 24 h repletion. Root $[NH_4^+]_i$ (closed square) increased rapidly in the first hour from 2.21 to 6.48 μmol g^{-1}FW and increased only slightly to 7.13 μmol g^{-1}FW during the next 23 h of NH_4^+ repletion (Fig. 6B). By contrast, depletion of G1000 plants in G2 medium increased $^{13}NH_4^+$ influx only very slightly during the first hours. Then influx increased rapidly from 0.72 to 7.29 μmol g^{-1}FW h^{-1} (open circle in Fig. 23A). During the depletion in G2 medium, the $[NH_4^+]_i$ of G1000 plants (open square) decreased gradually from 6.35 to a value similar to that of G2 at steady-state, 2.36 μmol g^{-1}FW by 12 h of depletion. During the next 12 h, there was only a small further decrease of $[NH_4^+]_i$ (Fig. 23B).

The changes of tissue amino acids present different patterns for plants undergoing nitrogen depletion or repletion. During the repletion process, G2 plants were exposed to 1000 μM NH_4^+ for up to 24 h. The total

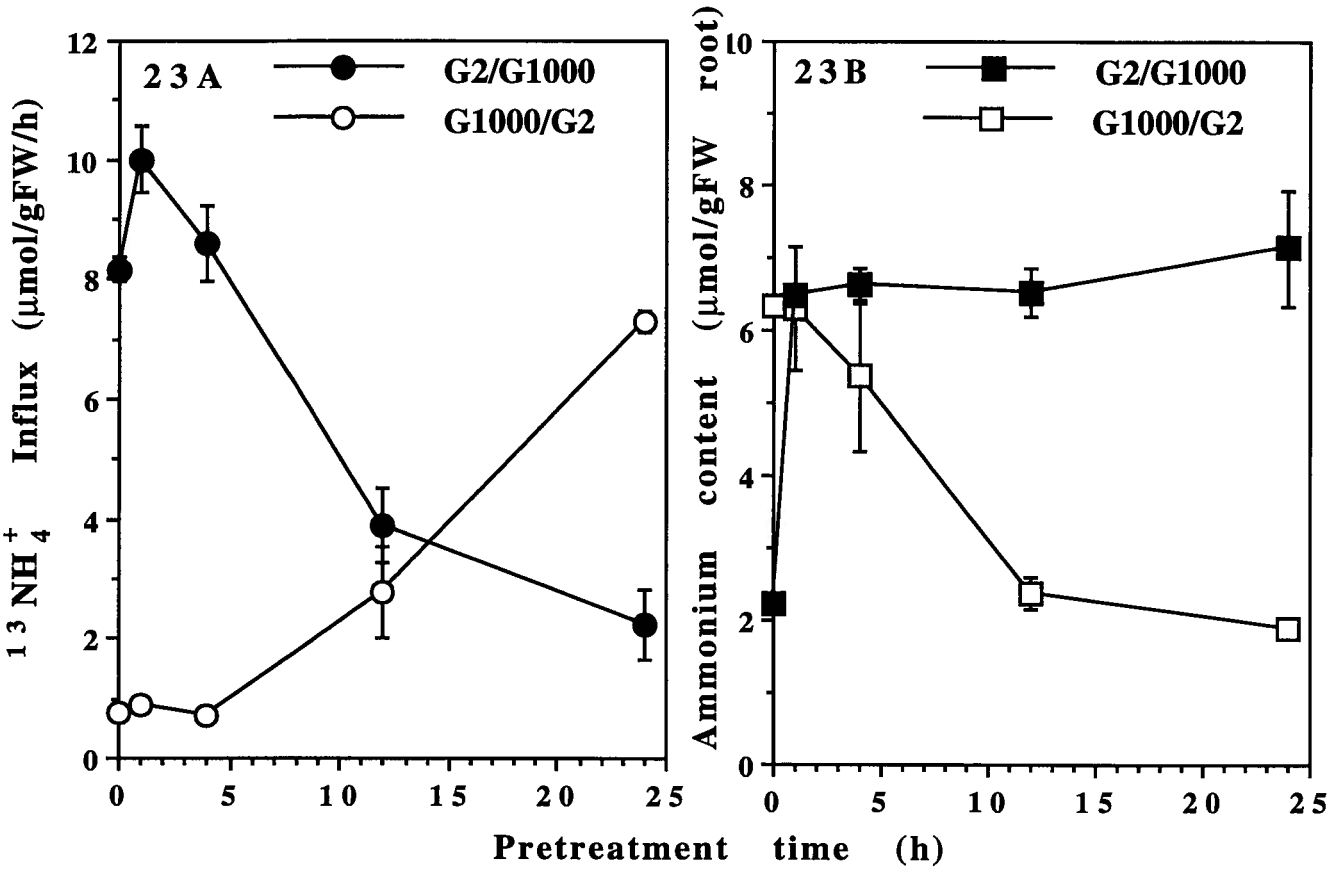


Figure 23. $^{13}NH_4^+$ influx (23A) and internal ammonium content (23B) of repleted G2 or depleted G1000 roots. 23A: $^{13}NH_4^+$ influxes of G2 or G1000 roots, after pretreatment for 1, 4, 12 and 24 h in G1000 or G2 medium, respectively, were measured in 100 µM $^{13}NH_4^+$-labeled solution. 23B: Internal ammonium content of the same roots. Each datum point is the mean of 6 replicates and the vertical bar represents the standard error.

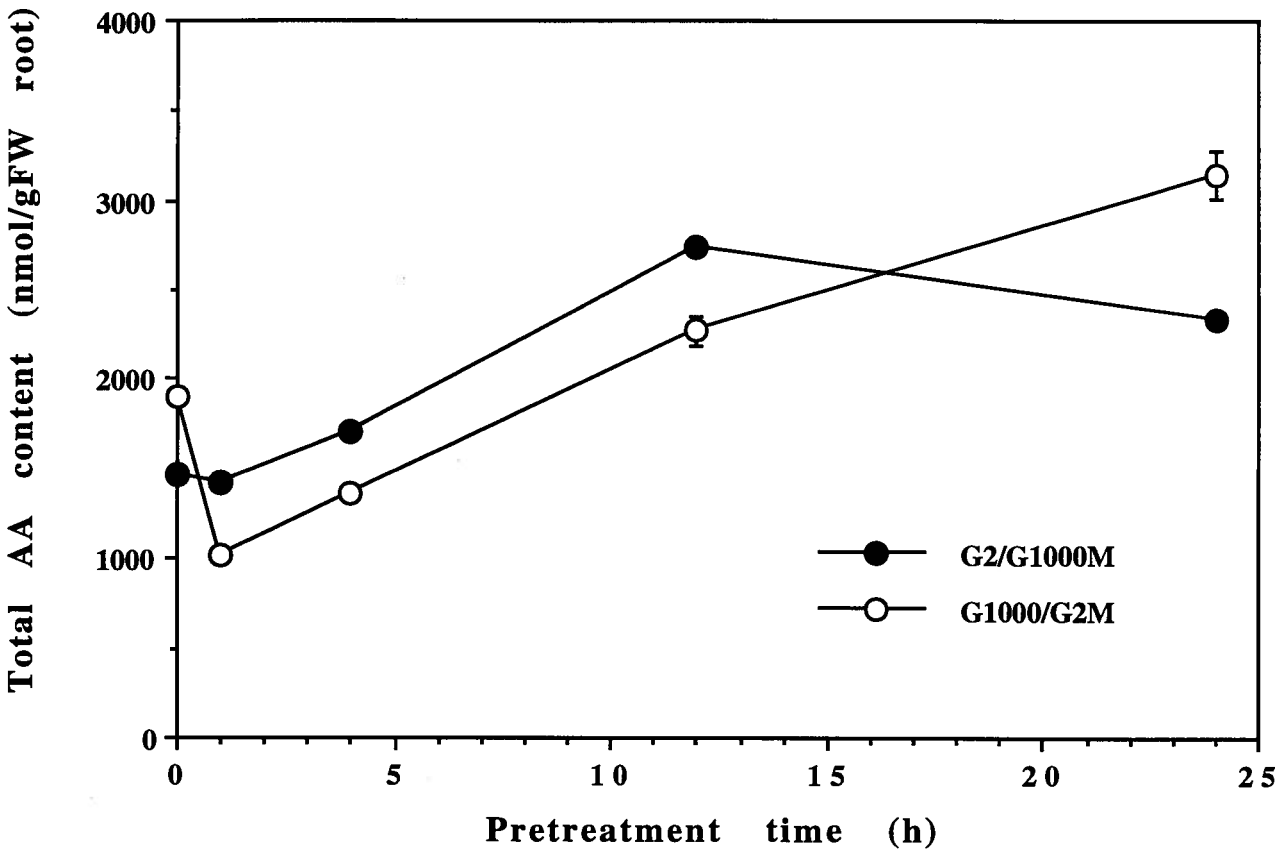


Figure 24. Total $[AA]_i$ of repleted G2 or depleted G1000 roots. Total $[AA]_i$ of G2 or G1000 roots were assayed after pretreatment for 1, 4, 12 and 24 h in G1000 or G2 medium, respectively. Each datum point is the mean of 6 replicates and the vertical bar represents the standard error (± se).

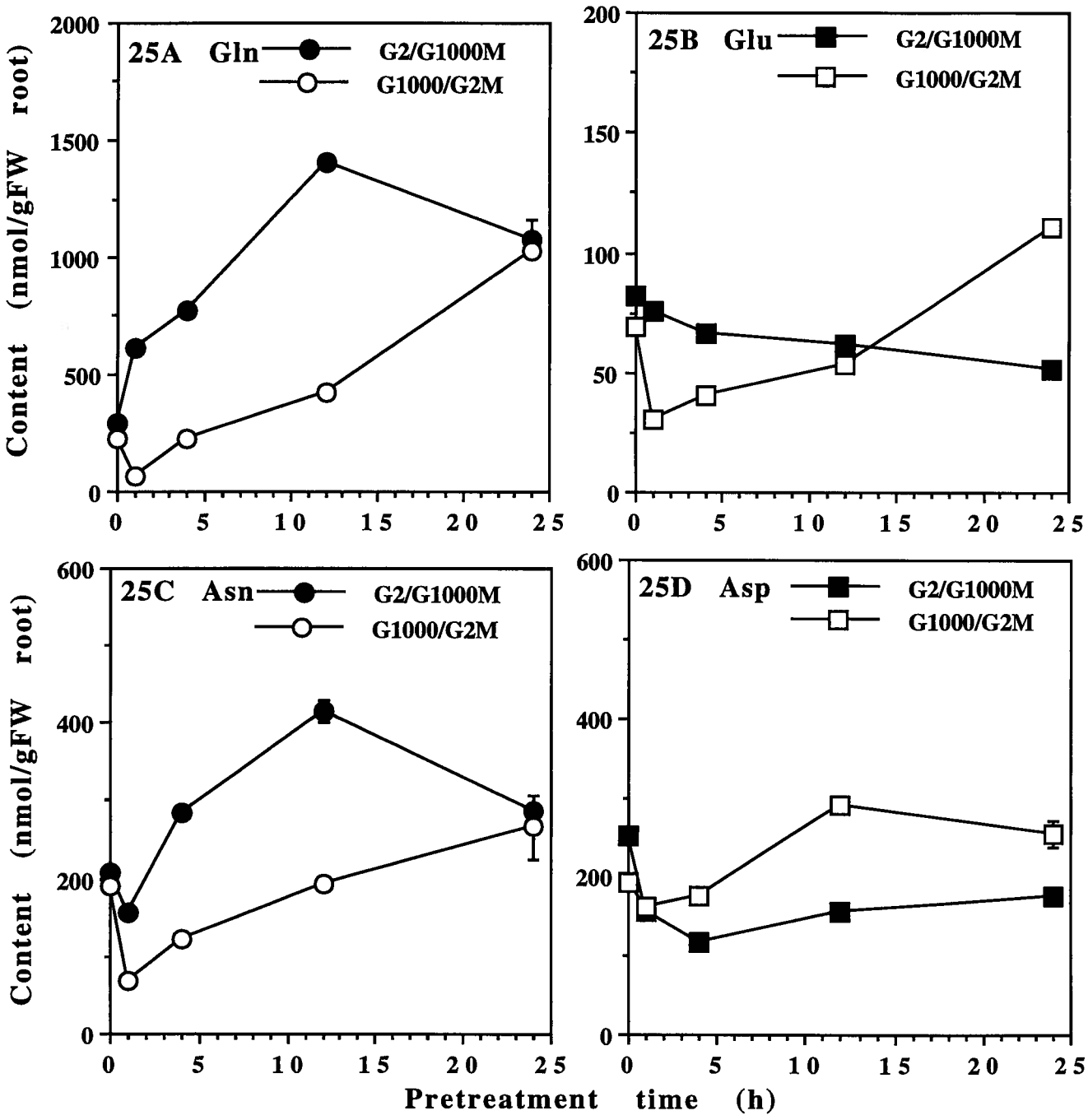


Figure 25. Tissue amide or amino acid contents of repleted G2 or depleted G1000 roots. After pretreatment for 1, 4, 12 and 24 h in G1000 or G2 media, respectively, the amino acid contents of G2 and G1000 roots were assayed. 25A for $[Gln]_i$; 25B for $[Glu]_i$; 25C for $[Asn]_i$,; 25D for $[Asp]_i$. Each datum point is the mean of 6 replicates and the vertical bar represents the standard error (± se).

$[AA]_i$ increased during 12 h of repletion and stayed at more or less the same level during the next 12 h (Fig. 24). The content of Gln (Fig. 25A, closed circles) changed in the same pattern as the total $[AA]_i$ but the Glu content (Fig. 25B, closed circle) decreased continuously during NH_4^+ repletion. Although the reduction of $[Glu]_i$ was 37%, $[Gln]_i$ increased 372% during 24 h of repletion. In contrast, $[Asn]_i$ decreased by about 24% during the first hour, then it increased nearly 39% of the initial level in the next 12 h (Fig. 25C, closed circles). $[Asp]_i$ was reduced (49%) in G2 roots during the first 4 h (Fig. 8D, closed squares), after which it increased slightly. When G1000 plants were depleted in G2 medium, total $[AA]_i$ as well as the four major amino acids decreased rapidly for the first hour (Figs. 24, 25A~D, open symbols). This is interesting because despite big changes in these [amino acid], influx changed little. After that, the $[AA]_i$, $[Gln]_i$, $[Glu]_i$, $[Asn]_i$, and $[Asp]_i$ increased 65%, 353%, 61%, 40% and 31%, respectively, within 23 h of commencing the depletion process.

6.3.2. Experiment II. Effects of MSX

Short periods (< 12 h) of MSX treatment increased $^{13}NH_4^+$ influx of G2 roots (closed circles in Fig. 26) from 8.17 to 16.93 μmol $g^{-1}FW$ h^{-1}, but longer (12 - 24 h) exposures reduced influx slightly, to 12.64 μmol $g^{-1}FW$ h^{-1}. During 24 h pretreatment of G2 plants in G1000+MSX, $^{13}NH_4^+$ influxes (open squares) remained essentially constant at about 10 μmol $g^{-1}FW$ h^{-1} and were lower than those of in G2+MSX (closed circles). Likewise, G1000 plants, pretreated in G2+MSX or G1000+MSX media, exhibited very low $^{13}NH_4^+$ influx values (closed and open squares) which remained essentially constant for the duration of the experiment. Fluxes of G1000 plants were

significantly lower than in G2 plants in G2+MSX or G1000+MSX (compare open to closed symbols in Fig. 26).

For G2 plants pretreated in G2+MSX, root $[NH_4^+]_i$ increased rapidly from 2.21 to 7.19 at the first hour and remained at the same level for the remainder of the experiment (closed circles in Fig. 27A), but pretreatment in G1000+MSX caused root $[NH_4^+]_i$ to increase rapidly from 2.21 to 8.49 μmol $g^{-1}FW$ during the first hour, reaching a value of 9.35 after 24 h repletion (closed squares in Fig. 27B). G1000 plants possessed a higher initial $[NH_4^+]_i$ (6.35 μmol $g^{-1}FW$) (Figs. 27A and 27B), which continuously increased to 8.57 μmol $g^{-1}FW$ after 24 h during treatment of G1000+MSX medium. Root $[NH_4^+]_i$ in G1000 plants treated in G2+MSX declined gradually from 7.36 at 1 h to 5.77 between 4 and 24 h (open circles in Fig. 27A). The increment of $[NH_4^+]_i$ in MSX treated plants varied with prior NH_4^+ provision during growth and additional depletion or repletion treatments (Figs. 27A and 27B). During the first hour, the $[NH_4^+]_i$ of G2 plants increased 230% in G2+MSX medium and 320% in G1000+MSX medium. The $[NH_4^+]_i$ of G1000 plants increased 35% in G1000+MSX medium, and 16% during the same time period in G2+MSX medium, the latter then decreased to 9% after 24 h.

The total $[AA]_i$ of G2 or G1000 plants in the four treatments pretreated with 1 mM MSX, remained at similar levels, respectively, over the 24 h period (Figs. 28A and 28B). G1000 plants (open symbols) had a higher total $[AA]_i$ than G2 plants (closed symbols). Both plants showed a small increase in the G1000+MSX treatment (Fig. 28B). Pretreatment in G2+MSX, caused the $[Gln]_i$ of G2 roots to decline at the first hour but no further changes were observed during the remainder of the experiment (Fig. 29A, closed circles). The opposite effect was observed in G1000+MSX

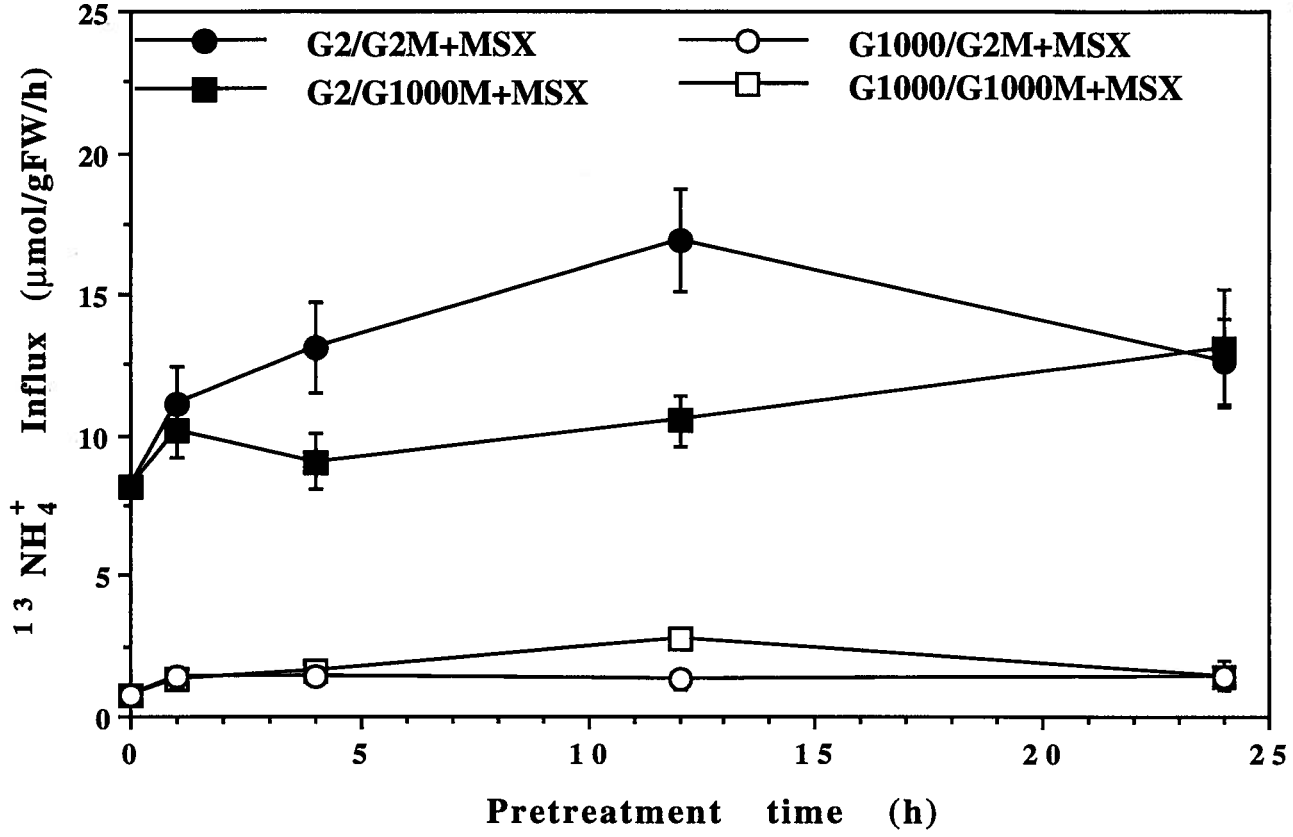


Figure 26. Effect of MSX pretreatment on $^{13}NH_4^+$ influx of rice roots. G2 (closed symbols) or G1000 plants (open symbols) were pretreated with 10 mM MSX for a maximum duration of 24 h including 0, 1, 4, 12, and 24 h in G2+MSX medium (open or closed circles) and in G1000+MSX medium (open or closed squares), respectively. The influxes were measured in 100 μM $^{13}NH_4^+$-labeled solution without MSX. Each datum point is the mean of 6 replicates and the vertical bar represents the standard error (± se).

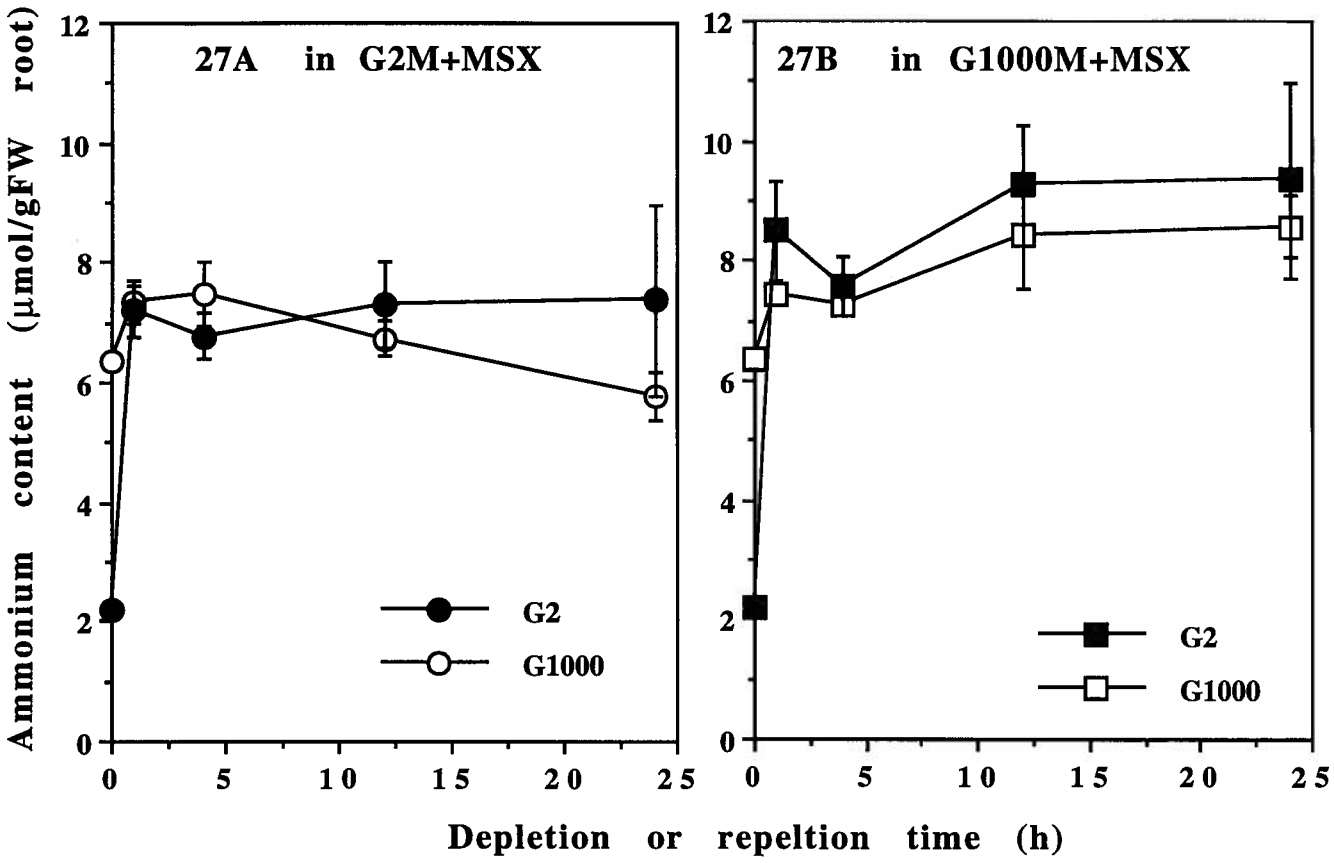


Figure 27. Effect of MSX on internal ammonium content of rice roots. The pretreatments and symbols are same as in Fig. 26. Each datum point is the mean of 6 replicates and the vertical bar represents the standard error (± se).

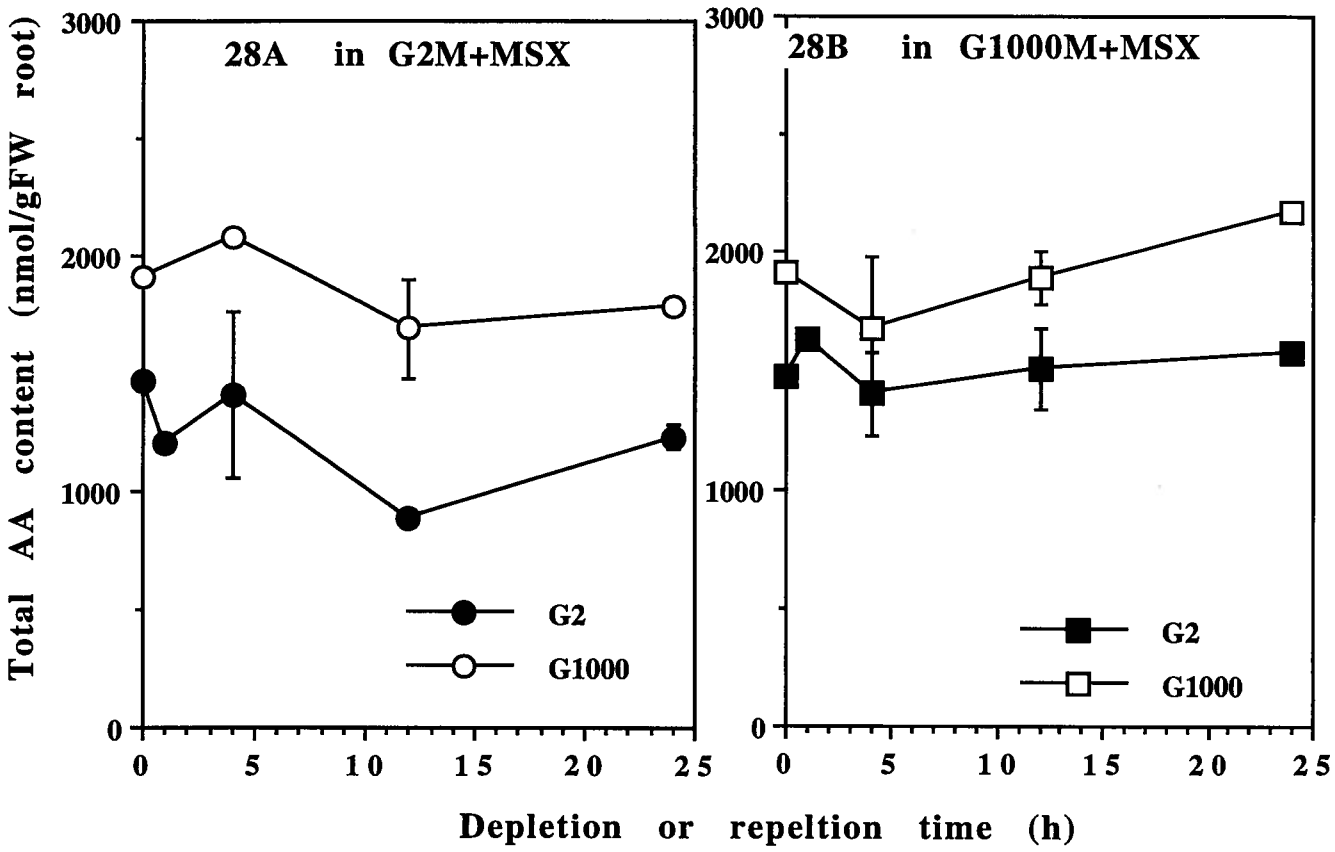


Figure 28. Effect of MSX on total $[AA]_i$ of rice roots. The pretreatments and symbols are same as in Fig. 9. Figures 11A and 11B are for the plants pretreated in G2+MSX medium and in G1000+MSX medium, respectively. Each datum point is the mean of 6 replicates and the vertical bar represents the standard error (± se).

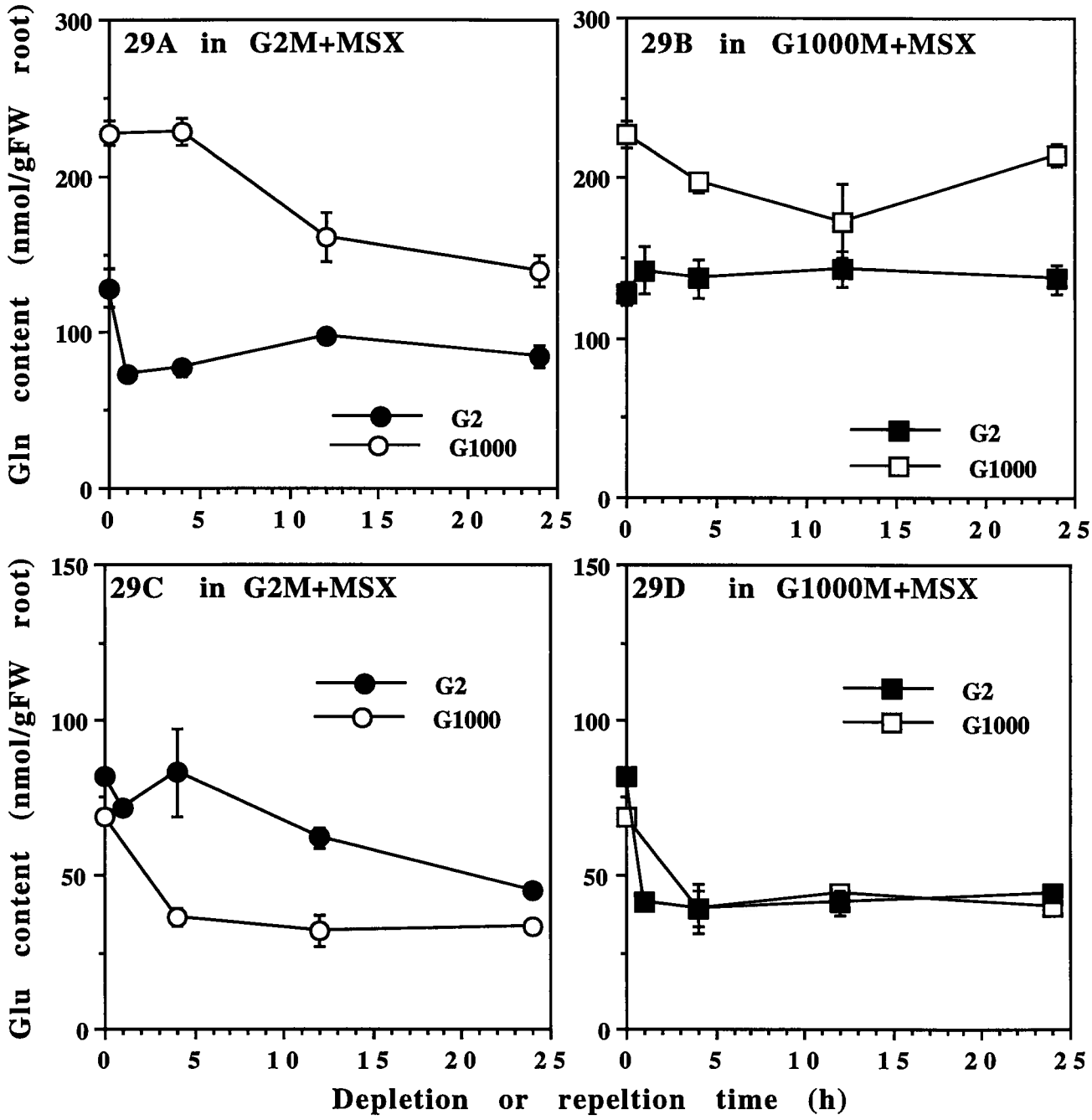


Figure 29. Effect of MSX on amide or amino acid content of rice roots. The pretreatments and symbols are the same as in Fig. 26. Figures 29A, 29C, 29E, 29G is for $[Gln]_i$, $[Glu]_i$, $[Asn]_i$, and $[Asp]_i$ of plants pretreated in G2+MSX medium, respectively. Figs. 29B, 29D, 29F, 29H is for $[Gln]_i$, $[Glu]_i$, $[Asn]_i$, and $[Asp]_i$ of plants pretreated in G1000+MSX medium, respectively.

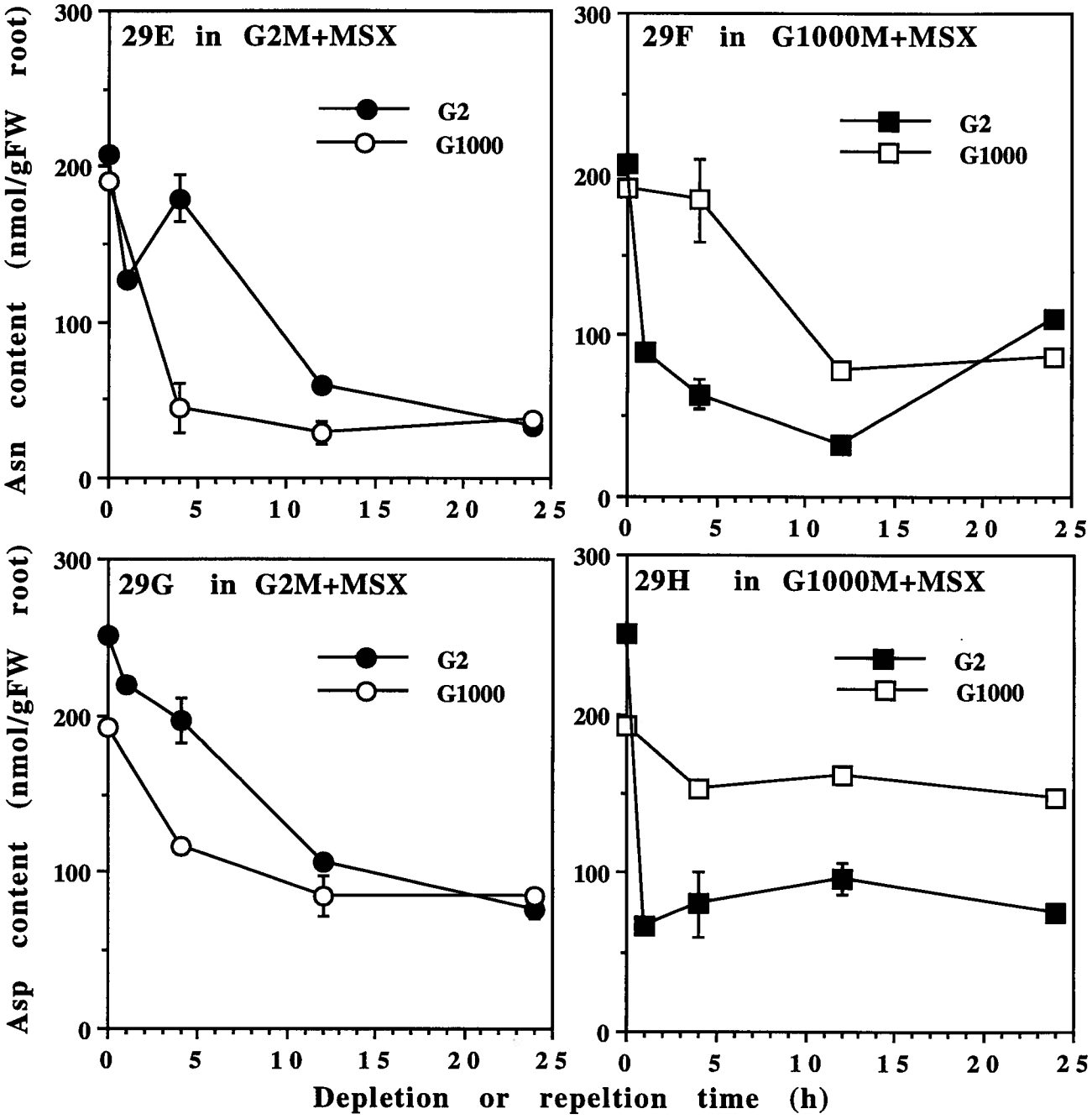


Figure 29. (Continued).

(Fig. 29B, closed squares). The $[Gln]_i$ of G1000 roots was reduced more in G2+MSX (Fig. 29A, open circles) than in G1000+MSX (Fig. 29B, open squares). In the latter medium, Gln recovered slightly after 24 h pretreatment (Fig. 29B). The levels of $[Glu]_i$ in roots declined rapidly within the first 4 h of pretreatment in G2+MSX and in G1000+MSX (Figs. 29C and 29D) except in the G2 plants treated in G2+MSX, in that it took a longer time to achieve the same reduction (Fig. 29C, closed circles). The $[Asn]_i$ and $[Asp]_i$ of G2 roots were also significantly reduced in all four pretreatments (Figs. 29E~H). A similar extent of reduction of $[Asn]_i$ was reached in a shorter time period when G1000 plants were pretreated with MSX in either repletion with or depletion of NH_4^+ (open circles in Fig. 29C and open squares in Fig. 29D) whereas the change of $[Asp]_i$ was more gradually in G2+MSX (Fig. 29G) than in G1000+MSX (Fig. 29H); in the latter treatment the reduction occurred within 4 h of pretreatment.

6.3.3. Experiment III. Effects of exogenous amino acids

Pretreatment of G100 roots with 10 mM glutamine significantly reduced $^{13}NH_4^+$ influx at all concentrations tested (Fig. 30). Assays of $[NH_4^+]_i$ revealed that glutamine pretreatment was associated with higher $[NH_4^+]_i$ (6.2 ± 0.5 μmol g^{-1} FW) than those pretreated without glutamine (2.3 ± 0.8 μmol g^{-1}FW). The 18 h pretreatment in 10 mM Gln raised the contents of Gln, Glu, and Asp near 4 times and Asn 7 times (Figs. 31A and 31B).

The interaction of exogenous amino acids and nitrogen status were also investigated. When G2 plants were treated with either 10 mM $[Gln]_o$ or $[Glu]_o$ for 18 h, $^{13}NH_4^+$ influxes were significantly reduced (from 8.94

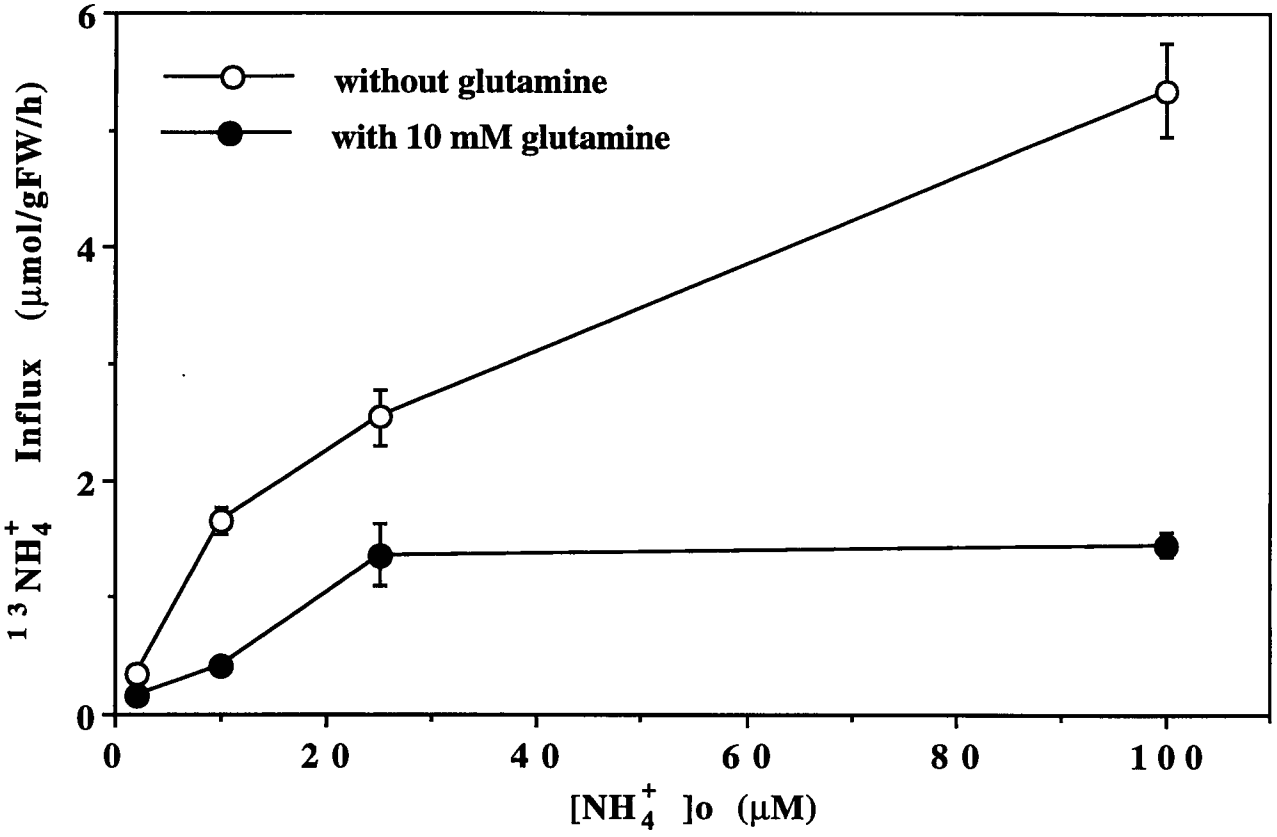


Figure 30. Effect of exogenous glutamine on $^{13}NH_4^+$ influx of roots. G100 plants were pretreated in G100 medium with or without 10 mM glutamine for 16 h before measuring $^{13}NH_4^+$ influx. $^{13}NH_4^+$ influxes were measured in 2, 10, 25 and 100 μM $^{13}NH_4^+$-labeled solution without glutamine. Each datum point is the mean of 6 replicates and the vertical bar represents the standard error (± se).

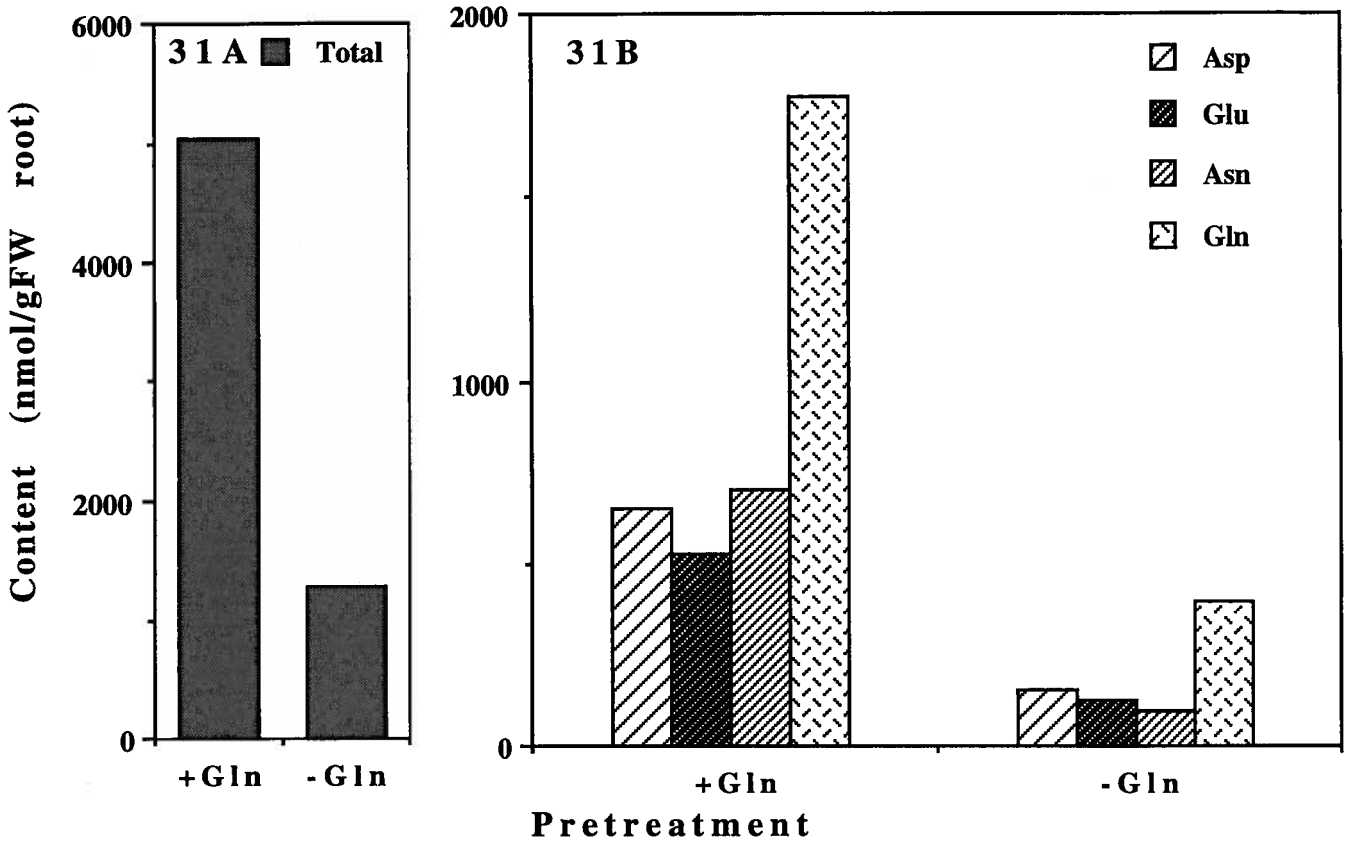


Figure 31. Effect of exogenous glutamine on the contents of amides and amino acids of root tissues. The pretreatments are same as in Fig. 30. Fig. 31A is total $[AA]_i$ and Fig. 31B is $[Gln]_i$, $[Glu]_i$, $[Asn]_i$, and $[Asp]_i$. Each datum point is the mean of 6 replicates and the vertical bar represents the standard error (± se).

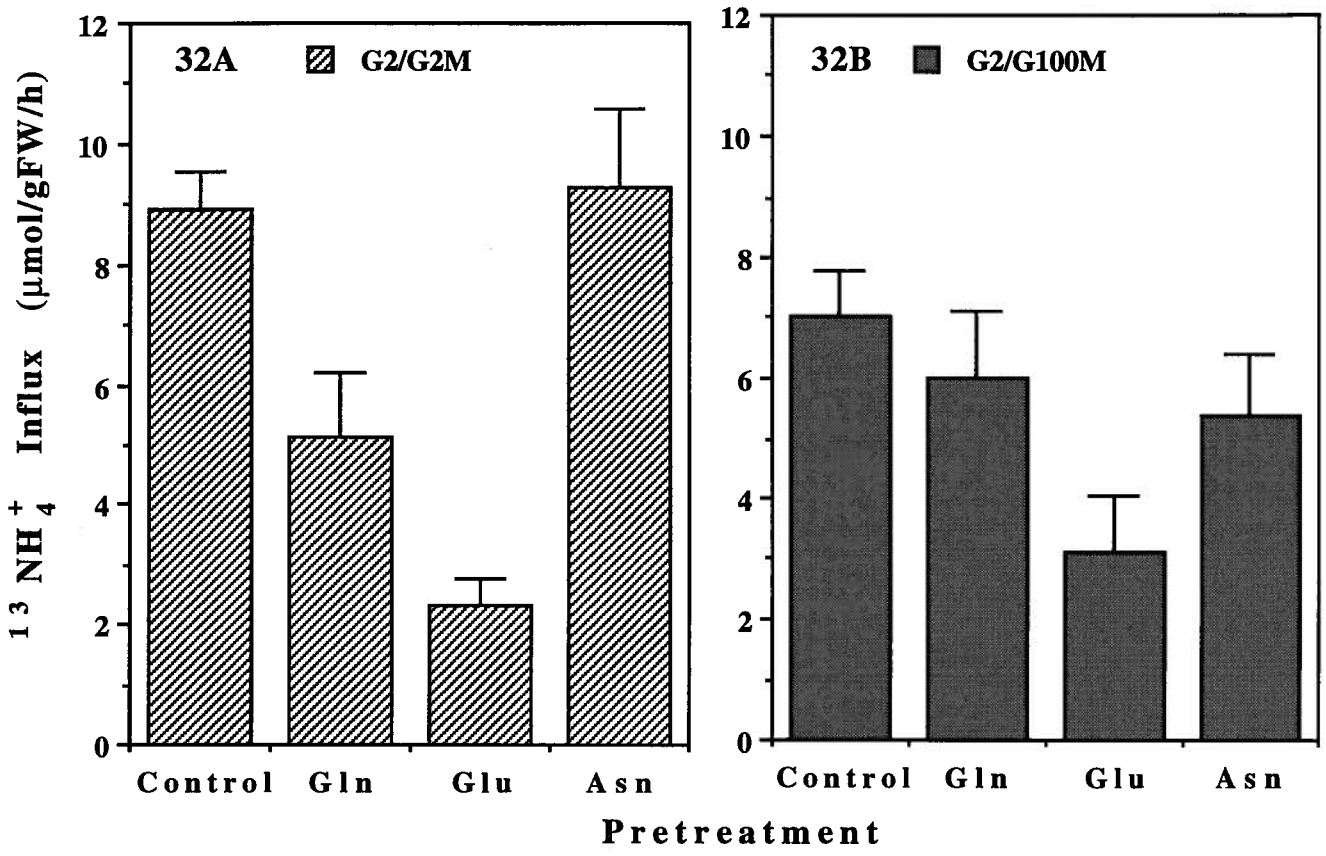


Figure 32. Effect of exogenous amides and amino acid on $^{13}NH_4^+$ influx. G2 plants were pretreated in G2 medium (Fig. 32A) or G100 medium (Fig. 32B) in the presence of 10 mM of either Gln, or Glu, or Asn for 6 h. The influxes were measured in 100 μM $^{13}NH_4^+$-labeled solution. Each datum point is the mean of 6 replicates and the vertical bar represents the standard error (±se).

μmol g^{-1} FW h^{-1} of the control to 5.12 and 2.30, respectively, Fig. 32A). No significant reduction of $^{13}NH_4^+$ influx occurred as a result of Asn pretreatment (Figs. 32A, B). Comparisons of pretreatments in G2 medium and G100 medium for G2 plants, revealed that the higher concentration of NH_4^+ in the latter medium led to a reduction of $^{13}NH_4^+$ influxes of the control and the Asn-pretreated plants from 8.94 and 9.26 μmol g^{-1} FW h^{-1} (Fig. 32A) down to 7.04 and 5.36 μmol g^{-1} FW h^{-1}, respectively (Fig. 32B). The combination of 100 μM $[NH_4^+]_o$ and 10 mM $[Gln]_o$ or $[Glu]_o$ (Fig. 32A) failed to reduce $^{13}NH_4^+$ influx further than the pretreatments of 2 μM $[NH_4^+]_o$ and 10 mM $[Gln]_o$ or $[Glu]_o$ (Fig. 32A). Pretreatments with exogenous amides or amino acids increased $[NH_4^+]_i$ from 1.1 to 3.6 - 5.5 μmol g^{-1} FW at low external NH_4^+ conditions (G2 medium) (Fig. 33A). In G100 treatment, internal $[NH_4^+]$ was higher for the Glu pretreatment, followed by the control, and the pretreatments with Gln and Asn (Fig. 33B).

Total AA concentrations were significantly higher for plants pretreated in G100 medium than in G2 medium (Fig. 34). In both cases, the total AA was higher in the pretreatments of Glu and Asn (Fig. 34B). When exogenous amides or amino acids were provided during pretreatments, $[Gln]_i$ was highest in the Gln pretreatment (Figs. 35A and 36A), except for the $[Glu]_o$ pretreatment in G2 medium that had the highest $[Gln]_i$ (Fig. 34A). Compared to the control, the concentrations of Gln were doubled in both media. $[Glu]_i$ was highest in Gln pretreatments, followed by the Asn pretreatment (Figs. 35B and 36B). Both $[Asn]_i$ and $[Asp]_i$ were highest in the Asn pretreatment (Figs. 35C, 36C, 35D and 36D).

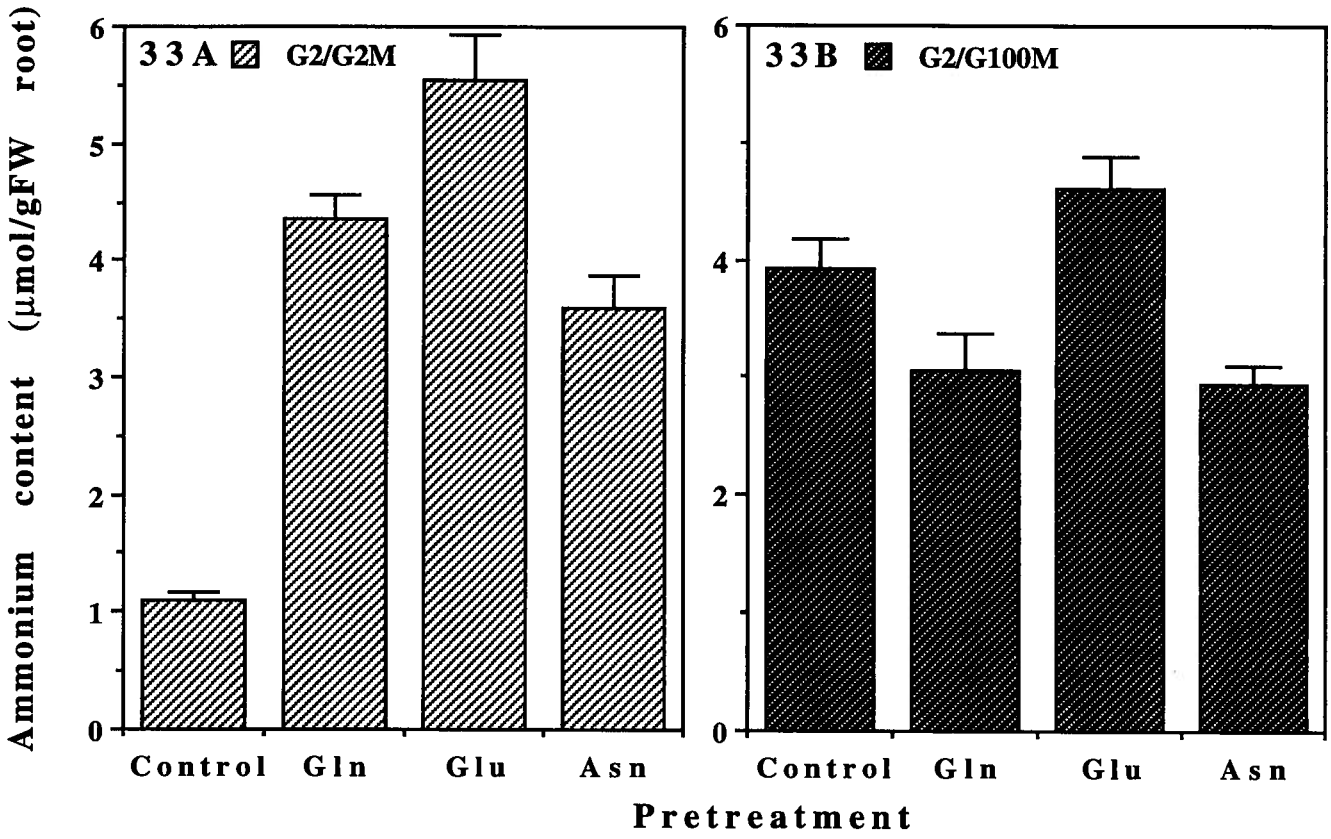


Figure 33. Effects of exogenous amides and amino acid on internal ammonium content. Details as in Fig. 32.

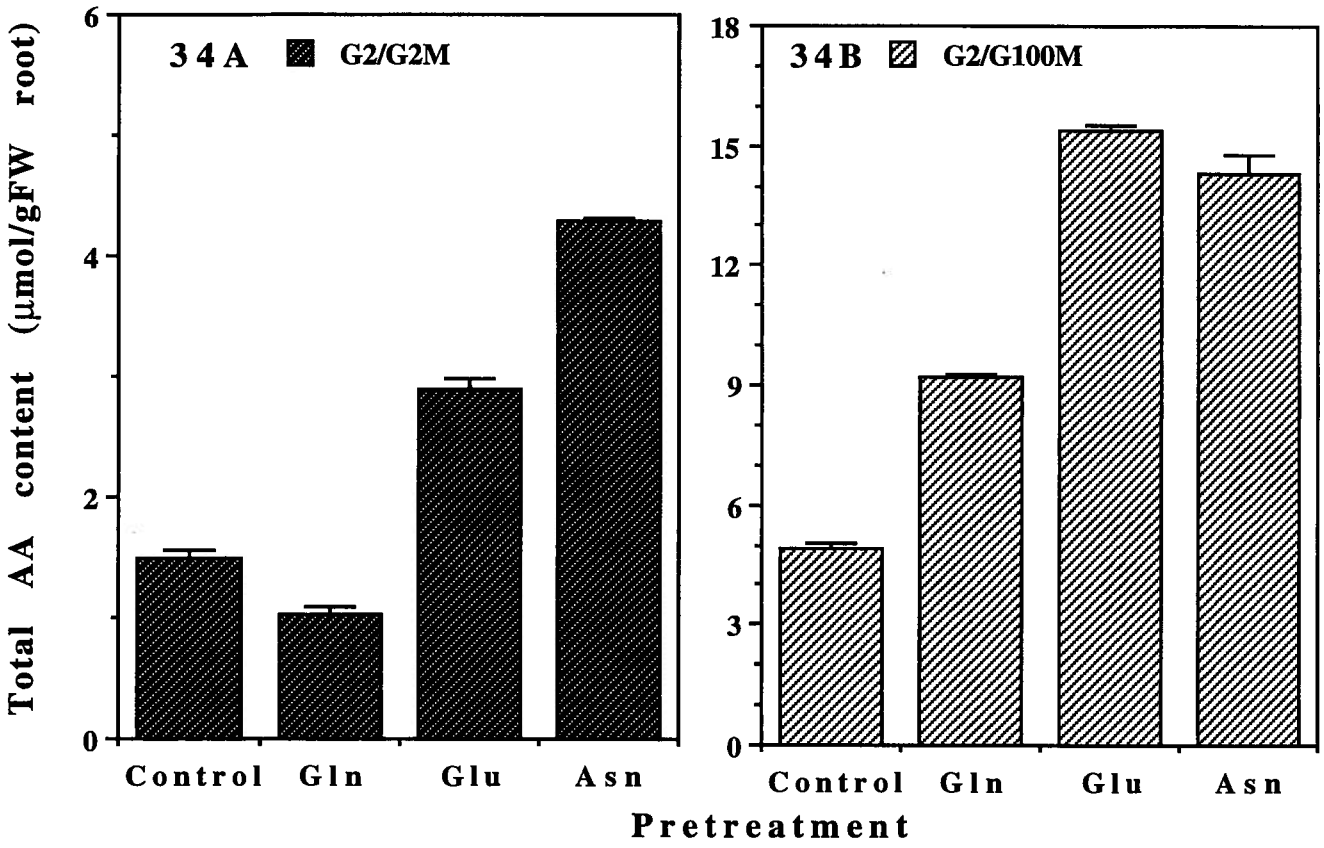


Figure 34. Effects of exogenous amides and amino acid on total amino acid content. Details as in Fig. 32.

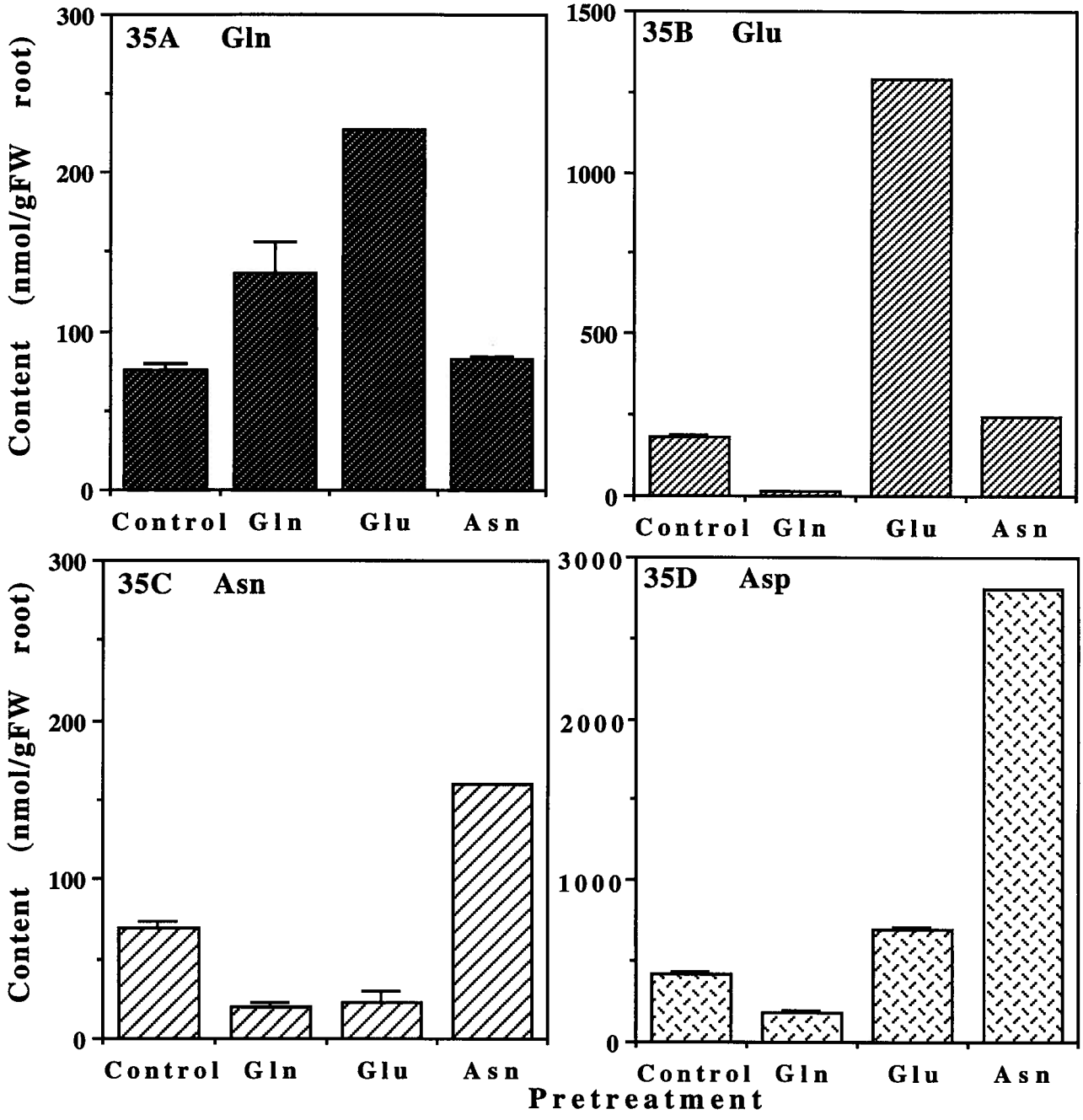


Figure 35. Effect of exogenous amides and amino acid on contents of amino acids in G2 roots. Pretreatments are same as in Fig. 32. Figs. 35A~D is for $[Gln]_i$, $[Glu]_i$, $[Asn]_i$, and $[Asp]_i$ of plants pretreated in G2 medium, respectively.

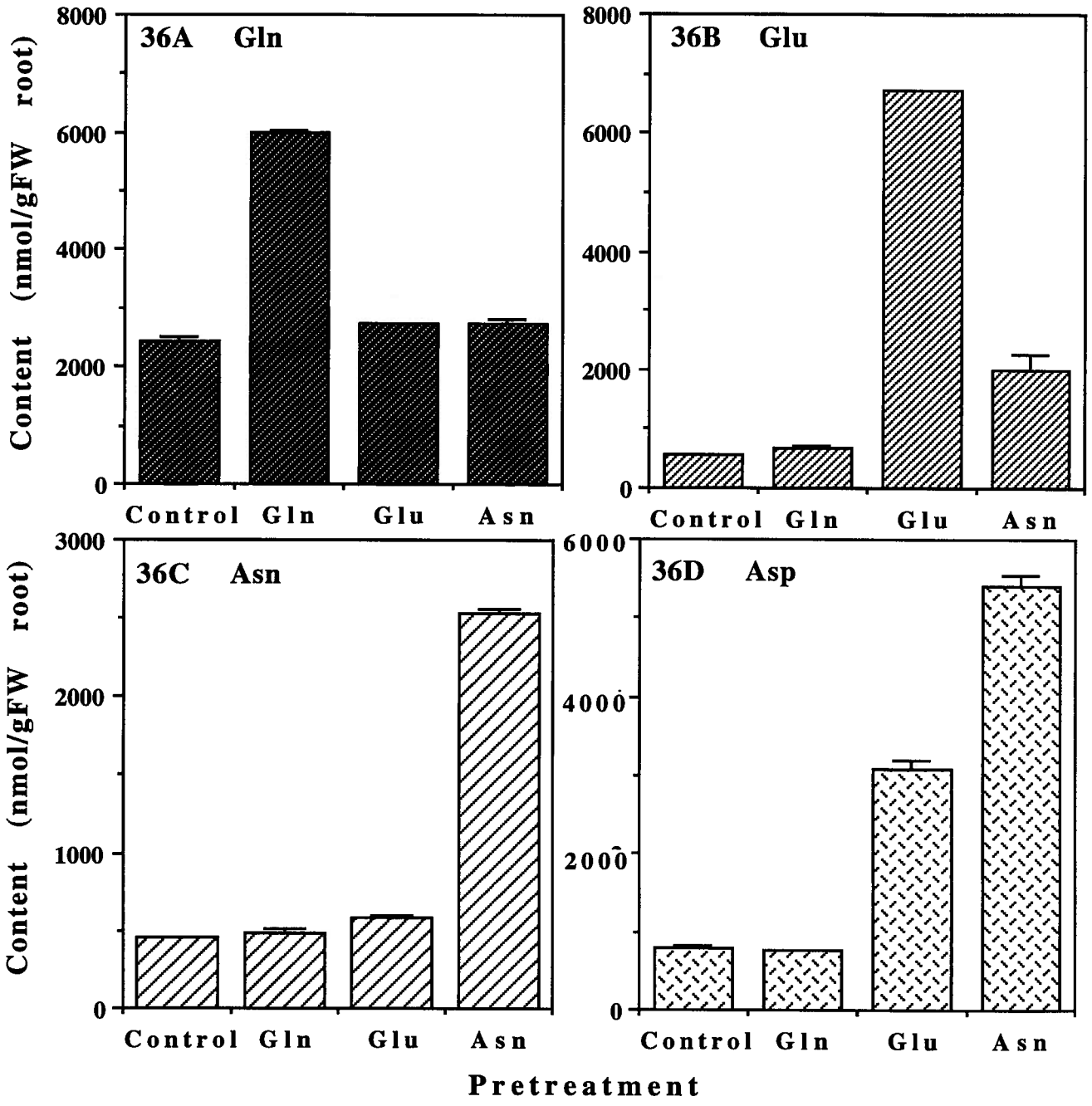


Figure 36. Effect of exogenous amides and amino acid on contents of amino acids in G100-pretreated roots. Pretreatments are same as in Fig. 32. Figs. 36A~D is for $[Gln]_i$, $[Glu]_i$, $[Asn]_i$, and $[Asp]_i$ of plants pretreated in G100 medium, respectively.

6.3.4. Experiment IV. Effects of selected inhibitors

G100 plants were treated with inhibitors of glutamine synthesis, MSX, glutamate synthesis, DON, and aminotransferases, AOA, for 16 h, respectively. The $^{13}NH_4^+$ influxes were measured in either 100 μM or 10 mM labeled $^{13}NH_4^+$ solution without inhibitors. The largest effect of the inhibitors of NH_4^+ assimilation was associated with AOA pretreatment (Fig. 36A). The $^{13}NH_4^+$ influx due to HATS (high affinity transport system) and LATS (low affinity transport system) were reduced by 68% and 32%, respectively (Figs. 37A and 37B). MSX reduced $^{13}NH_4^+$ influx by the LATS 25% and by the HATS 19%. DON treatment produced only a slight reduction of $^{13}NH_4^+$ influx (16% for LATS and 4% for HATS).

As can be seen in Fig. 38A, MSX significantly increased $[NH_4^+]_i$ almost 3 fold. The level of $[NH_4^+]_i$ was 1.9 times higher as a result of AOA pretreatment, while rice roots treated with DON actually had a lower $[NH_4^+]_i$ than the control. The total $[AA]_i$ was doubled by the AOA treatment (Fig. 38B). While slightly increased by MSX, the total $[AA]_i$ was greatly reduced by DON treatment. Looking at the four major amides and amino acids, (as shown in Figs. 39A, B, C, D), the pretreatment of AOA significantly increased all four, to a level which was at least double that of the control. There were no dramatic changes due to the MSX pretreatment. The four major amino acids were reduced to about half that of controls after treating plants with DON (Figs. 39A, B, C, D).

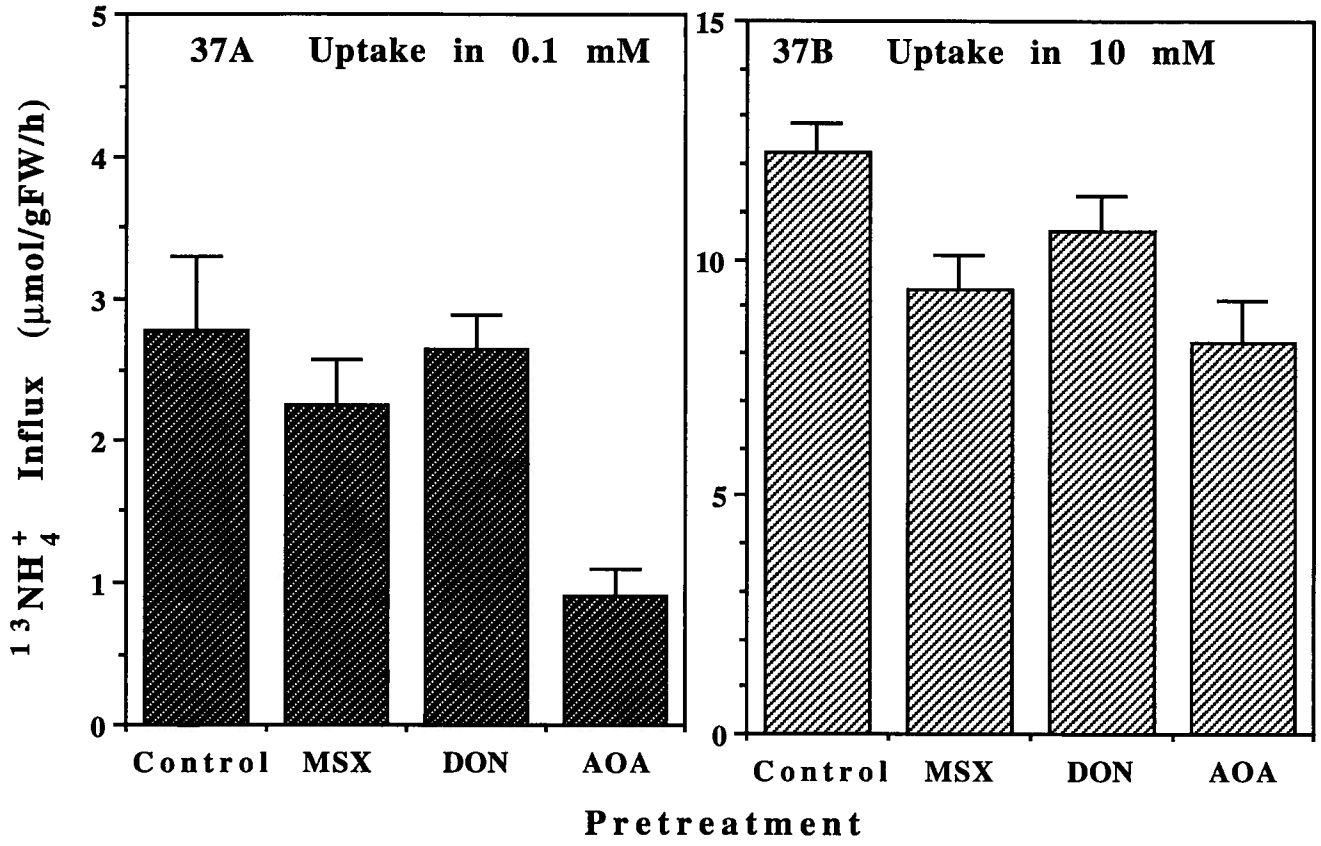


Figure 37. Effect of MSX, DON and AOA on $^{13}NH_4^+$ influx. G100 plants were pretreated with MSX, DON, and AOA for 16 h, respectively. The influxes were measured in either 100 µM (Fig. 37A) or 10 mM (Fig. 37B) labeled $^{13}NH_4^+$ solution without inhibitors. Each datum point is the mean of 6 replicates and the vertical bar is the standard error (± se).

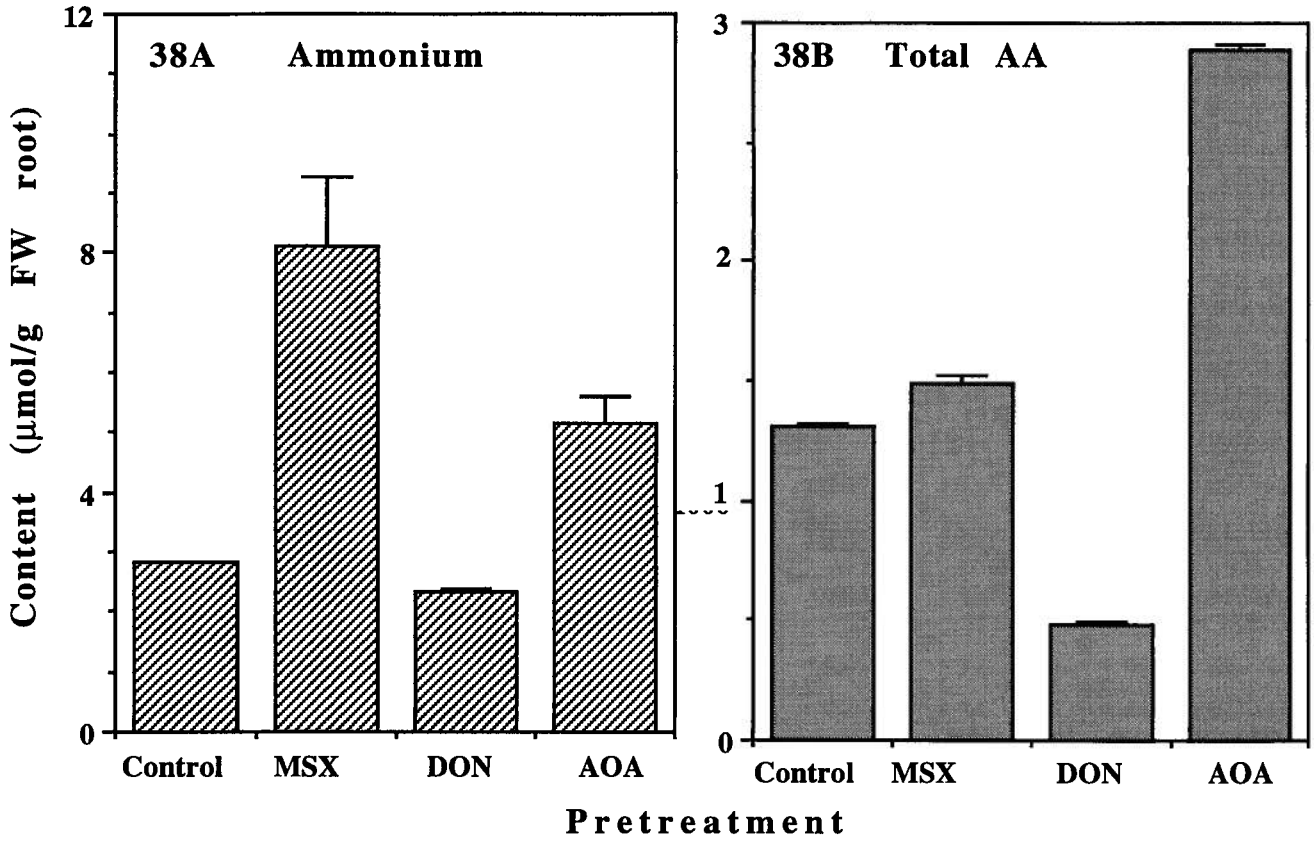


Figure 38. Effect of MSX, DON and AOA on internal ammonium and total amino acid content. Pretreatments are same as in Fig. 37. Fig. 38A is for internal ammonium and Fig. 38B is for total amino acid content.

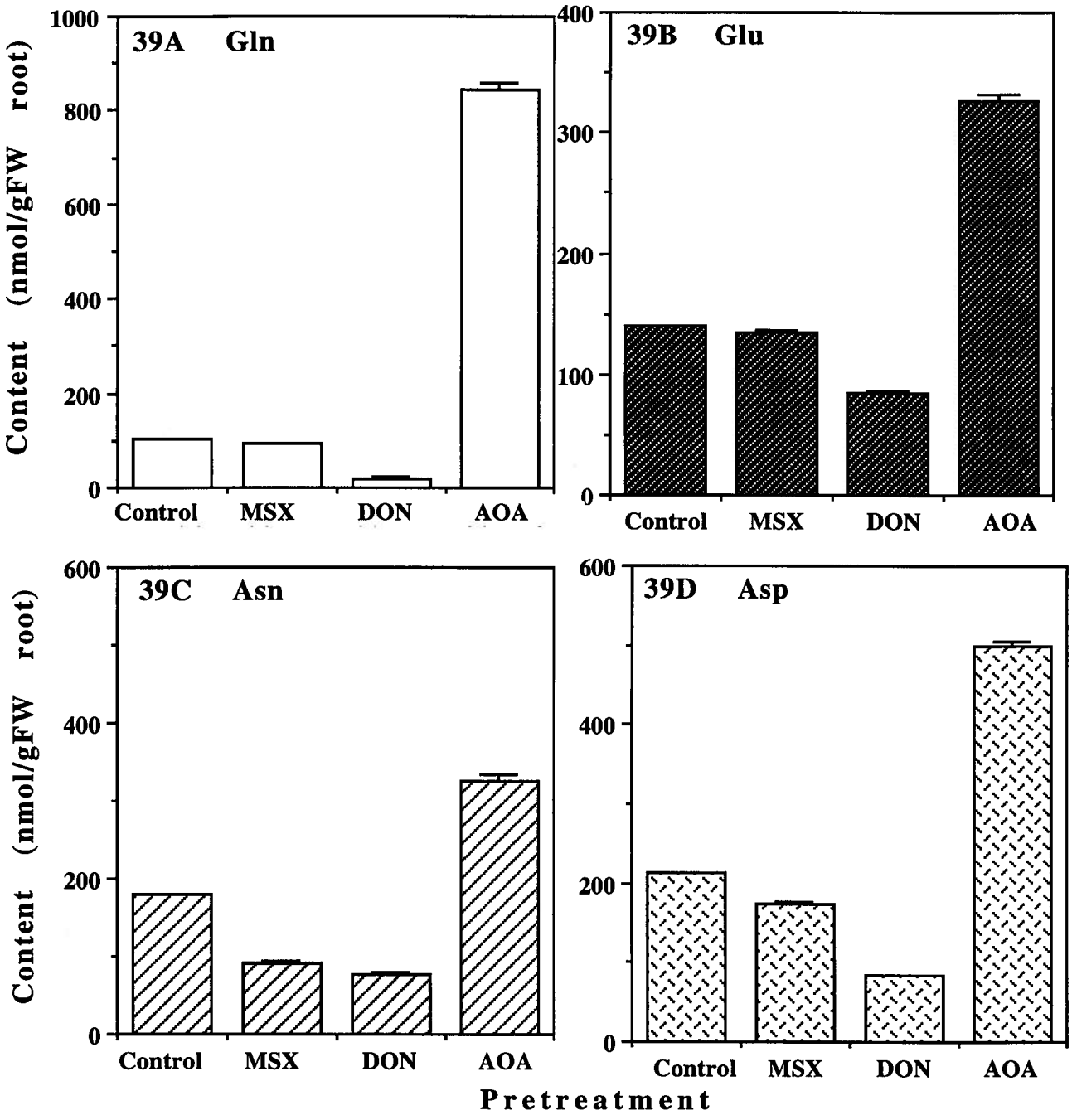


Figure 39. Effect of MSX, DON and AOA on major amino acid contents. Pretreatments are same as in Fig. 37. Figs. 39A~D is for $[Gln]_i$, $[Glu]_i$, $[Asn]_i$, and $[Asp]_i$, respectively. Each datum point is the mean of 6 replicates and the vertical bar is the standard error (± se).

6.4 DISCUSSION

6.4.1. Negative feedback on NH_4^+ uptake by NH_4^+ assimilates

NH_4^+ uptake is probably regulated continuously in response to the N status of the plant, but it is not clear how this is achieved. Increase in ammonium influx upon nitrogen limitation and decrease in influx as cell nitrogen status rises have commonly been observed (McCarthy and Goldman, 1979; Pelley and Bannister, 1979; Smith, 1982; Ullrich et al. 1984; Holtel and Kleiner, 1985; Clarkson, 1986; Lee and Rudge, 1986; Morgan and Jackson, 1988a, 1988b; Clarkson and Lüttge, 1991). Feedback inhibition of NH_4^+ uptake by nitrogenous effectors has been implicated in organisms like *Lemna*, algae, yeast and higher plants (Kleiner, 1985; Ullrich et al., 1984; Pelley and Bannister, 1979; MacFarlane and Smith, 1982; Wiame et al., 1985; Wright and Syrett, 1983; Thomas and Harrison, 1985; Clarkson and Lüttge, 1991).

The product(s) of ammonium assimilation have been proposed to act as the negative feedback factors for the NH_4^+ uptake process (Cook and Anthony, 1978b; Breteler and Siegerist, 1984; Wiame et al., 1985; Revilla et al., 1986; Lee and Rudge, 1986; Morgan and Jackson, 1988a). In the review by Clarkson and Lüttge (1991) a central role for glutamine in regulating the uptake of N by fungi and microalgae was presented. Glutamine or asparagine are the low molecular weight N-containing compounds stored or translocated by plants in the family of *Poaceae* (***Gramineae***) (Marschner, 1986). Lee and Rudge (1986) showed sizable increases in NH_4^+ uptake by barley following N-depletion, and the increased capacity for NH_4^+ uptake was inversely related to the reduced-N

status of the root tissue. In tobacco cells cultured on nitrate, urea, or ammonium, Gln is the first major organic product of assimilation of $^{13}NH_4^+$ (Skokout et al., 1978). It is also true for rice, because glutamine and glutamate were the primary products of ammonium assimilation in rice roots (Arima and Kumazawa, 1977). However the studies by Lee et al., (1992) and by several other workers (summarized in Clarkson and Lüttge, 1991) showed that other amino acids may participate in the regulation of N uptake.

In the present study, evidence supporting a central role for glutamine or other amino acids in controlling NH_4^+ influx was equivocal. When plants were maintained at 2 μM or 1000 μM NH_4^+ respectively, $^{13}NH_4^+$ influx was inversely correlated with $[Gln]_i$ (closed symbols compared to open symbols in Figs. 29A and 29B). Likewise, when the internal concentrations of Gln and other amino acids were increased by pretreatment with Glu, $^{13}NH_4^+$ influx declined (Figs. 30, 31B and 35A). The results indicated that Glu had an inhibitory effect on $^{13}NH_4^+$ influx, greater than Gln or Asn (Figs. 32A and 36A). This point was supported by the results of the AOA treatment. After treating plants with AOA, under the conditions of the present study there was a significant increase of $[Gln]_i$ (Fig. 39A), $[Glu]_i$ (Fig. 39B), $[Asn]_i$ (Fig. 39C), and $[Asp]_i$ (Fig. 39D). This increment was associated with a significant reduction of $^{13}NH_4^+$ influx (Fig. 20A). It must be pointed out that the above mentioned reductions of $^{13}NH_4^+$ influx in rice also coincided with a significant increase of $[NH_4^+]_i$ (Figs. 33A and 38A). Pretreatment with 10 mM Gln doubled the $[NH_4^+]_i$ from 2.30 to 6.10 μmol $g^{-1}FW$ (also in Fig. 33A) and decreased $^{13}NH_4^+$ influx.

In the depletion experiment shown in Fig. 23A transfer of G1000 plants to G2 solution failed to increase NH_4^+ influx until 4 h had elapsed. Yet, the amino acid analysis indicated strong reduction of total $[AA]_i$ and [Gln], [Glu] and [Asp] (Figs. 24, 25A~D). Strong reductions of amino acids were not correlated with $^{13}NH_4^+$ influx. Therefore, it is not entirely clear which N derivative is responsible for limiting influx.

Although applying organic N to the growth media has been found to increase crop yield (Mori et al., 1977; Mori and Uchino, 1977), the treatment of organic N suppresses the uptake of inorganic N. For example, maize roots pretreated with Gln or Asn exhibited reduced net uptake of NH_4^+ and NO_3^- (Lee et al., 1992). The uptake of $^{15}NO_3^-$ by barley roots was depressed by pretreatment with Arg and His (Mori et al., 1979). It was suggested that transport activity for ammonium was controlled by intracellular rather then extracellular metabolites (Jayakumar and Barner, 1984).

6.4.2. Effect of MSX: reduced amino acid pool

MSX inhibited the activity of glutamine synthetase in plant roots, and stopped the ^{15}N labeling of free amino acids, particularly glutamine and glutamate in roots of barley or rice (Arima and Kumazawa, 1977; Lewis et al., 1983). Preventing the assimilation of newly absorbed NH_4^+ or releasing NH_4^+ from the catabolism of internal N-containing compounds rapidly increased the NH_4^+ concentration in roots (Arima and Kumazawa, 1977; Lewis et al, 1983; Lee et al., 1992). Two major effects are expected: the amino acid pool is reduced and NH_4^+ pool is increased. After treating with MSX, tissue $[Gln]_i$ is typically decreased (Steward and Rhode, 1976; Fentem

et al., 1983a, 1983b) and consequently the amide donor to Asn synthesis is decreased, since the concentrations of Gln and Asn closely correlated (Lee et al., 1992). When products of ammonium assimilation were reduced by treatment of MSX, NH_4^+ influx was increased (Jackson et al., 1993), though NO_3^- influx was not stimulated (Lee et al., 1992).

MSX increased the cytoplasmic ammonium concentration in root tissue of rice (Arima and Kumazawa, 1977), Datura (Probyn and Lewism 1979), barley (Lewis et al., 1983; Fentem et al., 1983b; Morgan and Jackson, 1988a, 1988b); wheat (Morgan and Jackson, 1988a, 1988b), maize (Lee and Ratcliffe, 1991; Lee et al., 1992). A ten fold increment of the cytoplasmic pool was reported in maize roots compared to the control (Lee and Ratcliffe, 1991; Lee and Ayling, 1993). This increase is due to two effects: (a) the assimilation of NH_4^+ into amino acids is blocked, and (b) the production of NH_4^+ from breakdown of amino derivatives remains unaffected. It has been claimed that release of NH_4^+ from this degradation path occurs at a rate which is 50% higher than the rate of NH_4^+ influx (Jackson et al., 1993). As a result, ammonium appeared in the xylem sap (Lee and Ratcliffe, 1991) and net NH_4^+ efflux was increased substantially (Morgan and Jackson, 1988a). Arima and Kumazawa (1974, 1975, 1976, 1977) proposed that most of the glutamine is synthesized adjacent to the outer membrane of plasma membrane of root cells, through which ammonium with a high ^{15}N abundance permeates from the external solution. MSX treatment might enlarge this ammonium compartment near the membrane.

Another explanation for the enhanced NH_4^+ influx by MSX treatment is that MSX enlarged the cytoplasmic and vacuolar NH_4^+ pools of root tissue several times (Jackson et al., 1993; Lee and Ayling, 1993). The enlarged

NH_4^+ pools in cell enhanced influx of $^{13}NH_4^+$ in maize and barley (Lee et al., 1992; Lee and Ayling, 1993). According to Lee and Ayling (1993) this resulted in a large value of NH_4^+ influx because what was measured under these circumstances was a true value of influx. By contrast, under 'normal' circumstances (they claim) even short $^{13}NH_4^+$ influx measurements are compromised by a significant efflux. The results of studies on rice and barley (Siddiqi et al., 1991; Wang et al., 1993a) repudiate this interpretation because the half-life of the cytoplasmic compartment is too long (7-8 min) and the efflux term is too small (10% - 30%) compared to influx (Wang et al., 1993a).

The results from this study show that, after treating plants with MSX, the concentrations of major amides and amino acids were all reduced to different extents (Figs. 29A-H), accompanied by an increased $[NH_4^+]_i$. The increment of $[NH_4^+]_i$ was varied with NH_4^+ provision and additional depletion or repletion treatments (Figs. 27A and 27B). However the treatment with MSX in this experiment failed to increase the $^{13}NH_4^+$ influxes of G1000 plants treated in either G2+MSX or G1000+MSX medium (open symbols in Fig. 26) compared to the effects of depletion or repletion by the same plants in the absence of MSX (Fig. 23A). However, G2 plants treated with G2+MSX conditions revealed a significant increase of influx (Fig. 26). When the same G2 plants were treated in G1000 medium plus MSX there was no decline of influx of the sort observed in the absence of MSX (Fig. 23A). This is consistent with an important role of amino N in down-regulating influx in low-N plants. The lack of an increased influx when G1000 plants were transferred to G2 medium with MSX (Fig. 26) argues that internal $[NH_4^+]$ is important in maintaining low NH_4^+ fluxes in high-N plants. This has also been claimed by Causin and Barneix (1993) in

wheat. Thus the results of these experiments indicated that both $[NH_4^+]_i$ and $[AA]_i$ may play important role in regulating NH_4^+ fluxes.

6.4.3. Effect of short-term N depletion

It has been recognized that the nitrogen (both ammonium and nitrate) uptake capacity of plant roots is enhanced when plants undergo nitrogen depletion (Humphries, 1951; Jackson et al., 1976; Clement et al., 1979; MacKown et al., 1981; Breteler and Nissen, 1982; Lee and Rudge, 1986; Ingemarsson et al., 1987; Oscarson et al., 1987; Teyker et al., 1988; Siddiqi et al., 1989; Jackson and Volk, 1992). NH_4^+ uptake shows a particularly strong response in several species, such as wheat (Tromp, 1962; Minotti et al., 1969; Jackson et al., 1976b; Morgan and Jackson, 1988a, 1988b), ryegrass (Lycklama, 1963), maize (Ivanko and Ingversenm, 1971; Lee et al., 1992), barley (Lee and Rudge, 1986), and oats (Morgan and Jackson, 1988a, 1988b).

In the present study, rice also responded to nitrogen depletion with enhanced NH_4^+ influx (Fig. 20). The short-term depletion of high NH_4^+-grown plants (G1000) in low N medium (G2 medium) stimulated NH_4^+ influx during the first 4 to 5 h of depletion. $^{13}NH_4^+$ influx remained high for the next 20 h, then declined to a relatively lower rate for the next 20 h of depletion (Fig. 20B). Similar rapid initial increases of NH_4^+ uptake were observed when plants were depleted of N for the first 0.25 and 1 h (Lycklama, 1963; Minotti et al., 1969; Breteler, 1975; Deane-Drummond, 1986; Goyal and Huffaker, 1986; Morgan and Jackson, 1988a, 1988b, 1989).

A likely explanation for this enhancement is the removal of a factor which exerts negative feedback regulation on NH_4^+ uptake. Both NH_4^+ and its primary assimilate were suggested as such factors in uptake regulation (Breteler and Siegerist, 1984; Revilla et al., 1986; Lee and Rudge, 1986; Morgan and Jackson, 1988a). Another explanation for this enhancement is due to enhanced influx and reduced efflux (Morgan and Jackson, 1988a, 1988b). Substantial ammonium cycling occurred during net ammonium uptake (Jackson et al., 1993), yet plants grown in low N possess a low NH_4^+ efflux. For G2, G100 and G1000 plants at steady-state with respect to $[NH_4^+]_o$, the effluxes of NH_4^+ were 10%, 20% and 29%, respectively, of influx (Wang et al., 1993a). However, changes of these relatively small proportions may not account for the large increases of NH_4^+ uptake such as were observed in the present study.

In the present study, $[NH_4^+]_i$ was negatively correlated with influx during 4 h of depletion (Figs. 20B and 21B). It was also observed that the V_{max} for NH_4^+ influx was negatively correlated with internal NH_4^+ (Wang et al., 1993b). When plants were subjected to N depletion, the tissue content of NH_4^+ (Fig. 21B) dropped rapidly to lower levels and possibly resulted in a relief of N-suppression of the uptake process. $[NH_4^+]_c$ is a likely candidate for negative feedback regulation since the free NH_4^+ pools (cytoplasmic and vacuolar) will be drained in two opposite directions: efflux out of the tissue and metabolism into amino acids. In such a short time, $[NH_4^+]_c$ will be the first fraction to be drained to a minimum. Therefore internal NH_4^+ is a likely factor to exert a negative signal on NH_4^+ transport across the plasma membrane (also in section 6.4.5.).

It is generally believed that short periods of N depletion, less than 24 to 48 h, would not cause a decline of growth rate (Siddiqi et al., 1989;

Jackson and Volk, 1992). Though it was reported that enhanced uptake reached a maximum after 3 days of depletion when nitrogen stress was not severe enough to alter the RGR significantly (Lee and Rudge, 1986), longer N depletion may not sustain the maximum enhanced uptake rate due to possible adjustment of the RGR. For 8-d-old maize plants grown on 5 mM NO_3^-, NH_4^+ uptake rates increased steadily, and within 72 h of N-depletion, rates of NH_4^+ uptake initially increased followed by a decline and a subsequent increase (Jackson and Volk, 1992). This enhanced NH_4^+ uptake or NH_4^+ influx may be due to a relief of the uptake process from N-suppression. As suggested by Morgan and Jackson (1992), this type of response reflects the interplay of suppression by a product of ammonium assimilation, the accumulation of root ammonium and associated ammonium efflux, and a stimulation by ammonium of its own uptake.

6.4.4. Stimulated NH_4^+ influx after long-term N depletion

When N-depleted roots are first exposed to elevated levels of NH_4^+ there is an initial increase of NH_4^+ influx for the first few hours of exposure to NH_4^+ (Goyal and Huffaker, 1986; Morgan and Jackson, 1988a). The above workers observed a 25-35% increase of influx in wheat during the period from 2-10 h after exposure to NH_4^+; further exposure caused influx to decline. This phenomenon was found in wheat but not oat (Morgan and Jackson, 1988a). In the present study, an even greater effect was observed when G2 plants were repleted in G1000 medium. Within the first two hours repletion with NH_4^+, $^{13}NH_4^+$ influx increased rapidly from 11.10 μmol $g^{-1}FW$ h^{-1} to 31.97 μmol $g^{-1}FW$ h^{-1} (Fig. 19B). Then, influx dropped to the initial rate of about 10 μmol $g^{-1}FW$ h^{-1} after 8 h more repletion. A smaller stimulation can also be seen in Fig. 6A.

There are at least two possible explanations suggested for this phenomenon (Morgan and Jackson, 1988a). First, a second system for ammonium influx may be initiated (induced?) as N-depleted plants are exposed to ammonium for a short period before negative feedback become active. Another possibility is that there are two effectors (positive and negative) to regulate a single transport system. The positive effector could be NH_4^+ and the negative one may be a product of NH_4^+ assimilation (Morgan and Jackson, 1988a). Ammonium concentrations were related to the stimulation in influx whereas a product of ammonium assimilation was subsequently responsible for its reduction/inhibition (Wiame et al., 1985; Cook and Anthony, 1978a, 1978b).

The initial increase of NH_4^+ influx may be resulted from provision of N to synthesize more transporters that are sacrificed when plants are under N stress. In this sense, NH_4^+ would exert an effect as a source of N for transporters and also as a transport regulator. It was observed in a separate study (Fig. 43 in Chapter 7) that rice plants grown in low N and low K doubled their $^{86}Rb^+$ influx after preloading in 1 mM NH_4^+ for 2 h. In such a situation, it may be hypothesized that immediately after exposure to NH_4^+, more transporters are synthesized. This may not necessarily involve the synthesis of a different carrier for K^+ system. Re-supplying NH_4^+ provides the 'building blocks' to assemble more transporters to promote uptake and meet plant demand for N and K^+. Subsequently, negative feedback mechanisms begin to exert their regulation.

6.4.5. Negative feedback on $^{13}NH_4^+$ influx from internal NH_4^+

As discussed above, the theory of amides or amino acids as N uptake regulators can not explain all the observed results on the regulation of $^{13}NH_4^+$ influx. The data seem to indicate that internal NH_4^+ may able play a role in regulating NH_4^+ influx. It has been reported that ammonium transport is repressed by intracellular ammonium *per se* but not by its assimilates or *de novo* protein synthesis (Rai et al., 1986; Franco et al., 1987, 1988). The active, specific transport of $^{15}NH_4^+$ and ^{14}C-MA in both wild type and mutant cells of *Aspergillus nidulans* is regulated by the concentrations of internal ammonium (Pateman et al., 1973, 1974).

One of the major reported reasons for excluding NH_4^+ as a negative feedback factor was that there was not an exact parallel between root ammonium concentrations and net NH_4^+ influx (Lee and Rudge, 1986) or efflux (Morgan and Jackson, 1988a). Therefore endogenous NH_4^+ in roots appeared to exert no effect on uptake of either NH_4^+ (Lee and Rudge, 1986; Morgan and Jackson, 1988) or NO_3^- (Rufty et al., 1982a; Chaillou et al., 1991; Vessey et al., 1990a). Despite this claim by the above workers, there were negative correlations between NH_4^+ absorption and tissue concentration. It was reported that when plants were depleted of nitrate for a week, net NH_4^+ uptake was increased 5 to 10-fold (Morgan and Jackson, 1988a) "because of low internal NH_4^+ (1~2 μmol g^{-1})" (Morgan and Jackson, 1988a, 1988b, 1989). But this appears to agree that $[NH_4^+]_i$ is correlated (negatively) with NH_4^+ uptake. While there is a positive correlation between the N provision during growth and the internal content of NH_4^+ in root tissue, the V_{max} of $^{13}NH_4^+$ influx was negatively correlated with these two conditions (Wang et al., 1993a). Nitrogen depletion rapidly altered the N-status of the plants, particular the tissue

concentration (Vose and Bresse, 1964; Lee and Rudge, 1986). In the present study, within 4 to 12 hours of depletion of G1000 roots in G2 medium, $^{13}NH_4^+$ influxes increased and were closely correlated with decreases of internal NH_4^+ content (Figs. 20B, 21B, 23A, and 23B).

Lee and Ratcliffe (1991) argued that at steady-state, cytoplasmic ammonium concentration would be not in the millimolar range because the activity of GS was considerable higher than the uptake rate of NH_4^+ (4 μmol $g^{-1}FW$ h^{-1}). Glutamine synthetase from higher plants has a high affinity for ammonium (K_m ~20 μM) (Steward et al., 1980; Milflin and Lea, 1976). It would seem that if NH_4^+ is not accumulated to a certain level in the cytosol it would not be necessary to invoke a possible regulatory role for this N form. However, most reported estimates of cytoplasmic NH_4^+ concentration are in the millimolar range in roots of barley, maize, rice, onion, and wheat (Fentem et al., 1983b; Cooper and Clarkson, 1989; Macklon et al., 1990; Lee and Ratcliffe, 1991; Wang et al., 1993a). In the present study, the indirect estimation of cytoplasmic NH_4^+ concentrations would give 0.8 mM as the lowest value (Fig. 20A). However low concentrations in the cytoplasm may be due to its rapid movement into the vacuole. It was calculated that half of the total free NH_4^+ was in a 'storage pool' in the roots (Fentem et al., 1983a). In rice roots, it was estimated, that above 70% of NH_4^+ was stored in the root vacuoles (Wang et al., 1993a). The proportional distribution of newly absorbed NH_4^+ to N assimilation and to storage may depend on the balance between the gradient across the tonoplast and, the capacity of the GS/GOGAT system, which is probably influenced by whole plant N status. Since high external NH_4^+ repressed the activity of GS reversibly (Rhodes et al., 1976; Arima and Kumazawa, 1977) and NR (Siddiqi et al., 1993; King et al., 1993), NH_4^+ should have a role in regulating the NH_4^+ transport across plasma membrane but not the overall

N assimilation, which would include transport across the plasma membrane, metabolism, translocation and utilization (as discussed in section 6.4.6.).

Second, the rapid dispersion of NH_4^+ may be the reason it is so difficult to reveal the contribution of NH_4^+ to the regulation process. At low external $[NH_4^+]$, NH_4^+ entering across the plasma membrane is rapidly metabolized by GS/GOGAT at a rate that is potentially faster than influx (Lee and Ratcliffe, 1991), or is transferred to the vacuole for storage. There may be only limited opportunity for NH_4^+ *per se* to exert any direct regulation on NH_4^+ influx (transport step) under these conditions. Under conditions of elevated NH_4^+ supply, when the GS/GOGAT system and vacuole are relatively saturated, internal NH_4^+ may increase to a level which enables it to exert a negative feedback on the transport step. Under such condition, there may be a good correlation between $[NH_4^+]_i$ and accumulated primary products such as Gln. Ideally, the treatment with MSX blocks the assimilation of NH_4^+ into Gln therefore leading to increased $[NH_4^+]_i$ and decreased $[\text{amino acids}]_i$ in roots. As a result one might expect the influx to be increased. This was observed in the present study. Pretreatment of G1000 plants with MSX resulted in a decrease of all major primary products of NH_4^+ assimilation (open symbols in Figs. 29A~D). However these changes did not result in the enhanced $^{13}NH_4^+$ influxes as would be expected. Internal $[NH_4^+]$ remained at essentially the same level though there was a trend to reduce $[NH_4^+]_i$ in G2+MSX after 24 h pretreatment (open circles in Fig. 27A). This may be the reason $^{13}NH_4^+$ influxes increased during the first hour and remained at the same level thereafter (closed symbols in Fig. 26).

A comparison of the G2 plants treated with MSX in 2 mM or 1000 mM solutions (Fig. 26) revealed a significantly higher influx in the G2 plant treated in G2+MSX than in the G2 plant treated in G1000+MSX at 4 and 12 h. Yet the amino concentrations in the G2 plant treated in G1000+MSX showed no significant change during this period (Fig. 29). However $[NH_4^+]_i$ appeared to be higher in the G2 plant treated in G1000+MSX (Figs. 27A and 27B), consistent with an inhibitory effect of $[NH_4^+]_i$ on $^{13}NH_4^+$ influx whenever $[NH_4^+]_i$ is elevated; either by growth in high N condition or as a result of MSX treatment.

6.4.6. Cascade regulation system of nitrogen uptake

The process of NH_4^+ uptake may be sensitive to regulation from several signals, related to N status of the plant. These may include internal N pools (NH_4^+, NO_3^-, AA), the GS/GOGAT system, translocation (and recycling) and utilization. Clearly all these processes interact strongly. To imagine that only single cytosolic substrate (e.g. glutamine) might regulate the critical uptake step, may be naive. Therefore, there may be a cascade system with many levels of negative feedback regulation on NH_4^+ uptake. In addition to N signals, nitrogen (NH_4^+) uptake may be limited by the supply of carbohydrate from shoots (Kleiner, 1985). This could be considered as an important component of the regulation at the whole plant level. The ambient conditions such as light intensity and temperature will effect the production of carbohydrates. It was found, for example, that net NH_4^+ uptake rates oscillate between maximum and minimum with a periodicity co-ordinated with intervals of leaf emergence (Tolley and Raper, 1985; Tolley-Henry et al., 1988; Henry and Raper, 1989a; Rideout et al., 1994). At the time of emergence and early expansion of a new leaves

there is a requirement for large amount of nitrogen (Radin and Boyer, 1982; Steer et al., 1984), and carbohydrate (Turgeon, 1989). Therefore new leaves become the sink of photosynthate (Turgeon, 1989) and the flux of carbohydrate to roots is reduced. Nitrogen uptake depends on and competes (with other growth process) for soluble carbohydrate from the shoot (Raper et al., 1978; Lim et al., 1990; Henry and Raper, 1991), since carbohydrates provide metabolic energy for nitrogen uptake and translocation (Minotti and Jackson, 1970; Penning de Vries et al., 1974; Jackson et al., 1976). Translocation of carbohydrate from shoot to roots is responsive to concentration of carbohydrate in the shoot pool (Wann et al., 1978; Granato and Raper, 1989; Lim et al., 1990). Since NH_4^+ is assimilated rapidly and almost exclusively in roots as it is absorbed (Given, 1979; Chaillou et al., 1991), this source of carbon skeletons is equally important for NH_4^+ uptake and assimilation. It appears that regulation of both NH_4^+ and NO_3^- uptake at the whole-plant level is subject to common mechanisms that influence diverse processes within the root and are differentially affected by nitrogen stress (Rideout et al., 1994).

The next level of this cascade may be nitrogen assimilation and the major regulators responsible for controlling NH_4^+ uptake would be active inside root cells. These might include amides and some major amino acids (Pelley and Bannister, 1979; MacFarlane and Smith, 1982; Wright and Syrett, 1983; Ullrich, 1984; Kleiner, 1985; Thomas and Harrison, 1985; Wiame et al., 1985; Lee and Rudge, 1986; Morgan and Jackson, 1988b). As the primary product of NH_4^+ assimilation, glutamine is the primary candidate for negative effector (Cook and Anthony, 1978a, 1978b; Dubois and Grenson, 1979; Wiame et al., 1985). Within the N cycling of plants, the simultaneous movement of N-compounds from root to shoot, and from shoot to root (Cooper and Clarkson, 1989; Larsson et al., 1991) may enable

N absorption to be regulated to match the demand imposed by plant growth (Drew and Saker, 1975; Edwards and Barber, 1976). The concentrations of amides (Gln and Asn) in the roots will be the result of the balance between their synthesis from absorbed inorganic N (NH_4^+ or NO_3^-), their import via the phloem, and their export via the xylem (Lee et al., 1992).

Internal NH_4^+ has not been considered as a negative feedback effector for NH_4^+ uptake (Lee and Rudge, 1986; Morgan and Jackson, 1988a, 1988b; Raper et al., 1992), because it is claimed that there is no correlation between cumulative uptake of NH_4^+ and endogenous NH_4^+ in roots (Chaillou et al., 1991; Vessey et al., 1990a). One may consider NH_4^+ to be at the center of a vital process of uptake and metabolism. Unlike K^+, NH_4^+ will be rapidly consumed into amino acids within the root. Therefore, tissue [NH_4^+] is not an ideal indicator of N status. A second reason is that, it was observed by Morgan and Jackson (1988b) that during the first two days of N-deprivation, root NH_4^+ concentration and NH_4^+ uptake were closely correlated. After 5 d of N-deprivation, the root NH_4^+ concentrations were found increased slightly and the rate of NH_4^+ uptake was continued to increase. Based on present studies, NH_4^+ would be expected to be the negative effector when internal NH_4^+ levels increase beyond a certain level. Below this level one may assume that any free NH_4^+ would be immediately drawn into the metabolic process to meet the high demand for plant growth. There may be a critical nitrogen status below which the system is impaired and above which it is subject to repression and/or inhibition (Breiman and Barash, 1980).

It is proposed, therefore, that internal NH_4^+ represents a third level of control, operating whenever internal [NH_4^+] is elevated. The site(s) for

its putative effects may include the transport step at the plasma membrane, or the transcriptional level involving the genes coding for NH_4^+ transport.

In view of the different effects of internal NH_4^+ on NH_4^+ influx of N-repleted G2 plants and on N-depleted G1000 plants, it is possible that negative feedback regulation of NH_4^+ uptake may be facilitated by either NH_4^+ or its assimilates. In low N-grown roots the up-regulation of influx may be exerted through products of NH_4^+ assimilation, while in high N-grown roots, internal NH_4^+ may participate in the down-regulation of NH_4^+ uptake systems.

In the case of the up-regulation of $^{13}NH_4^+$ influx following transfer of G1000 plants to G2 medium (Fig. 20A), the $[NH_4^+]_i$ dropped during the first two hours of depletion (Fig. 20B) and then decreased gradually to a value similar to that of G2 plants at steady-state. I consider that cytoplasmic $[NH_4^+]$ may be the controlling effector here. This is based upon the following additional observations: first, similar negative correlations were found in the 24 h depletion experiment (Figs. 23A, 23B), however $^{13}NH_4^+$ influxes were negatively correlated with the $[NH_4^+]_i$ (Figs. 23A, 23B) but not the content of amides or amino acids (Figs. 24, 25A-D); Second, when the GS-GOGAT pathway was blocked by MSX, $^{13}NH_4^+$ influx remained at low rate (Fig. 26) due to higher $[NH_4^+]_i$ (Figs. 27A and 27B) despite a large decrease of four major amides and amino acids (Figs. 29A-H). Third, $^{13}NH_4^+$ influxes were different when G2 plants were pretreated with MSX for the same 24 h (Fig. 26), but transferred to either G2 or G1000 medium, which resulted in higher $[NH_4^+]_i$ for plants in G1000+MSX than in G2+MSX medium (Fig. 27). Since estimated half-life for cytoplasmic NH_4^+ exchange is <10 min (Wang et al., 1993a), it would be expected that this component

of internal [NH_4^+] would respond more dynamically to change of external [NH_4^+] than the vacuolar [NH_4^+].

In contrast, the observed declines of $^{13}NH_4^+$ influxes were related to high $[NH_4^+]_i$ and major amino acids (Figs. 23B, 24, and 25A-D). I interpreted this result to indicate that the decline of $^{13}NH_4^+$ influx normally observed when G2 plants are loaded in G1000 medium, depends upon products of NH_4^+ assimilation. This conclusion was supported by the results of glutamine pretreatment (Fig. 30), which reduced $^{13}NH_4^+$ influx at all concentrations tested. Further proof to this effect is provided by our amino acid analyses. Figure 25A and 25C show that transfer from G2 to G1000 medium caused $[Gln]_i$ and $[Asn]_i$ to increase several times while in the presence of MSX this increase was prevented (Figs. 29A and 29C). In addition the $^{13}NH_4^+$ influx was strongly correlated (negatively) with increased Gln, Glu, Asn, and Asp after treatment with AOA (Figs. 37A and 39A-D).

Chapter 7. INTERACTION BETWEEN K^+ AND NH_4^+

7.1. INTRODUCTION

Potassium uptake has been well studied in higher plants (Glass, 1975; 1976, 1978; Glass et al., 1981; Kochian and Lucas, 1982, 1988; Glass and Fernando, 1992). Likewise, the kinetics of ammonium transport have also been characterized (Becking, 1956; Fried et al., 1965; Ullrich et al., 1984; Wang et al., 1993a, 1993b). Despite the similarities between K^+ and NH_4^+, such as charge, hydrated ion diameter and some aspects of transport processes (Haynes and Goh, 1978), the interaction of these two cations is poorly understood .

The interaction between K^+ and NH_4^+ may be examined at different levels, such as the bioavailability in soils, effects on plant growth, and effect on plant roots' uptake/transport of these ions. Mutual beneficial effects of K and N on plant growth have often been described. An adequate K^+ supply has been shown to enhance NH_4^+ uptake and assimilation (Ajayi et al., 1970; Barker and Lachman, 1986; Scherer and MacKown, 1987) and is very important for nitrogen use efficiency. On the other hand, NH_4^+ may promote K^+ stress in rice (Noguchi and Sugamara, 1966) or reduce the K^+ concentration of plants (Claassen and Wilcox, 1974; Faizy, 1979; Lamond, 1979).

A number of studies have been carried out to investigate the interactions of K^+ and NH_4^+ at the transport level. In short-term experiments, the uptake of K^+ was significantly reduced by the presence of NH_4^+ in the uptake solution (Deane-Drummond and Glass, 1983b; Rosen

and Carlson, 1984; Morgan and Jackson, 1988). However the influence of K^+ on NH_4^+ uptake has not been consistent. In most cases, the uptake of NH_4^+ by plant roots has appeared to be independent of K^+ levels in the uptake solution and the K^+ status of the plants (Rufty et al., 1982; Rosen and Carlson, 1984; Scherer and MacKown, 1987). Nevertheless, Bange et al., (1965) reported that K^+ is capable of inhibiting NH_4^+ uptake in barley plants.

The objective of this study was to investigate the interactions between K^+ and NH_4^+ at the membrane transport step, and the influences of tissue K and N status on these ion fluxes, using $^{86}Rb^+$ and $^{13}NH_4^+$, respectively, as tracers.

7.2. METHODS AND MATERIALS

7.2.1. Plant growth and ^{13}N production

Section 2.2. Seed germination; section 2.3. Growth conditions; section 2.4. Provision of nutrients; section 2.5. Production of $^{13}NH_4^+$.

7.2.2. Experimental design

Three experimental variables were employed in this study involving N and K supply. These were (i) provision during three-week-growth periods or less as designated; (ii) pretreatment for up to three days prior to flux measurement; and (iii) presence in the uptake solutions. Test materials were 3-week-old rice seedlings. Each experiment was repeated

twice with three replicates. Both influxes of $^{13}NH_4^+$ and $^{86}Rb^+$ were calculated based on root fresh weight and 10 min uptake periods, except in experiment I, where the net fluxes of NH_4^+ and $^{86}Rb^+$ were calculated from 30 min uptake periods. Before and after transfer into or out of the radioactive isotopic labeled uptake solution, plant roots were prewashed and postwashed in an identical unlabeled solution for 5 and 3 min, respectively. These time periods were based on a previous study (Wang et al., 1993a, 1993b).

7.2.1.1. Experiment I: Effects of K^+ and NO_3^- in pretreatment, K^+ and NH_4^+ in uptake solutions on net K^+ and NH_4^+ fluxes.

Plants were grown in MJNS containing 200 μM K^+ plus 1.5 mM NO_3^- for 18 days, and were transferred to pretreatment solutions for three days. The pretreatments were MJNS with or without K and N (+K+N, -K+N, +K-N, -K-N) in which +K = 200 μM KH_2PO_4, -K = 100 μM $Ca(H_2PO_4)_2$; +N = 0.75 mM $Ca(NO_3)_2$; and -N = 0.75 mM $CaCl_2$. The $^{86}Rb^+$ influxes were measured from radioisotope-labeled MJNS (+K*+N, +K*-N) containing 200 μM K^+ with or without 200 μM NH_4^+. Net NH_4^+ fluxes were measured from MJNS containing 200 μM NH_4^+ with or without 200 μM K^+ (+K+N, -K+N).

7.2.1.2. Experiment II: Effects of NH_4^+ provision during growth and of K^+ and NH_4^+ in pretreatment and uptake solutions on $^{86}Rb^+$ (K^+) influxes.

Plants were grown in MJNS containing 200 μM K^+ plus 10, 50 or 100 μM NH_4^+, hereafter referred as G10, G50, or G100 plants, respectively. The plants were transplanted for three days to MJNS with or without additions of K and N, in which +K = 200 μM KH_2PO_4; -K = 100 μM $Ca(H_2PO_4)_2$; and +N = 10, 50 or 100 μM NH_4Cl; -N = 5, 25 or 50 μM $CaCl_2$ for G2, G10, or G100 plants, respectively. The $^{86}Rb^+$ influxes were measured from radioactive

isotopic labeled uptake solutions (MJNS containing 200 μM K^+ with or without 100 μM NH_4^+).

7.2.1.3. Experiment III: Effects of NH_4^+ provision during growth and presence in uptake solution upon influx isotherms for $^{86}Rb^+$ (K^+).

Plants were grown in four different growth media containing 2 or 100 μM NH_4^+ plus either 2 or 200 μM K^+, hereafter referred as G2/2, G2/200, G100/2, G100/200 plants, respectively. The $^{86}Rb^+$ influxes were measured in MJNS containing 2, 10, 50, 75, 100, 250 or 500 μM K^+, respectively, plus 2 μM NH_4^+ for G2/2 and G2/200 plants, or 100 μM NH_4^+ for G100/2 and G100/200 plants.

7.2.1.4. Experiment IV: Effects of NH_4^+ provision during growth and short-term pretreatment upon $^{86}Rb^+$ (K^+) influx.

Plants were pretreated for 0, 2, 4, 8, 24 h in 1 mM NH_4^+ plus 2 μM K^+ for G2/2 and G100/2 plants, or in 1 mM NH_4^+ plus 200 μM K^+ for G2/200 and G100/200 plants. $^{86}Rb^+$ influxes were measured during 10 min in uptake solution containing 100 μM NH_4^+ and 200 μM K^+.

7.2.1.5. Experiment V: Effect of NH_4^+ concentrations present in uptake solution upon influx isotherms for $^{86}Rb^+$ (K^+).

The $^{86}Rb^+$ influxes of G2/2, G2/200, G100/2, G100/200 plants were measured in MJNS containing 2, 25, 50, 100, or 200 μM K^+, plus 2, 25, 50, or 100 μM NH_4^+. The translocations of $^{86}Rb^+$ into plant shoots were also estimated based on the radioactivity recorded from plant shoots.

7.2.1.6. Experiment VI: Effects of K^+ provision during growth and presence in uptake solutions upon influx isotherms for $^{13}NH_4^+$.

The $^{13}NH_4^+$ influxes of G2/2, G2/200, G100/2, G100/200 plants were measured in uptake solutions (a) containing 2, 10, 50, 100, or 200 μM NH_4^+ plus either 0, or 200 μM K^+; (b) containing 100 μM or 10 mM NH_4^+ plus 2, 20, 200, or 2000 μM K^+.

7.3. RESULTS

7.3.1. **Experiment I:** Effects of K^+ and NO_3^- in pretreatment, K^+ and NH_4^+ in uptake solutions on net K^+ and NH_4^+ fluxes.

Pretreatment with NH_4^+ during three days prior to the uptake measurement generally increased $^{86}Rb^+$ (K^+) uptake. Only in -K+N, -K-N treatments was there no increase of $^{86}Rb^+$ uptake; all other treatments increased influx by 1.35 times (-K+N, -K-N) and 3.4 times (+K+N, +K-N) when NH_4^+ was absent from the uptake solution and by 1.85 times (+K+N, +K-N) when NH_4^+ was present (Table 13). Yet, the means for +K+N and -K+N were not significantly different at the 5% level of probability when NH_4^+ was present in the uptake solution. When NH_4^+ was absent, the means (3.29/0.96 for +K+N/+K-N and 8.72/6.44 for -K+N/-K-N) were statistically different at the 1% level.

The removal of K during pretreatment caused a much greater effect on $^{86}Rb^+$ accumulation, increasing $^{86}Rb^+$ (K^+) uptake by 2.65 and 6.7 times when it was absent from uptake solution, and by 5.25 and 10.2 times

Table 13. Net $^{86}Rb^+$ flux measured with or without ammonium. Rice plants were grown in MJNS containing 1.5 mM NO_3^- and pretreated 3 days in 4 different solutions with or without either 200 μM K^+ (+K or -K) or 1.5 mM NO_3^- (+N or -N). The net flux of $^{86}Rb^+$ was measured in the following uptake solutions: +K+N or +K-N (N = 200 μM NH_4^+ and K = 200 μM K^+) labeled with $^{86}RbCl$. Fluxes were calculated based on 30 min uptake periods.

Pretreatment	Uptake solution			
	+K+N		+K-N	
	($\mu mol\ g^{-1}FW\ h^{-1}$)			
+K+N	0.63 ± 0.07	d	3.29 ± 0.14	c
-K+N	3.31 ± 0.22	c	8.72 ± 0.53	a
+K-N	0.34 ± 0.02	d	0.96 ± 0.02	d
-K-N	3.47 ± 0.28	c	6.44 ± 0.28	b

* For comparing all possible pairs of treatment means (±se), Duncan's Multiple Range Test were performed, separately, on the data of net $^{86}Rb^+$ flux. Means having a common letter are not significantly different at the 5% significance level for small letter.

Table 14. Net NH_4^+ flux measured with or without potassium. Rice plants were grown in MJNS containing 1.5 mM NO_3^- and pretreated 3 days in 4 different solutions with or without either 200 μM K^+ (+K or -K) or 1.5 mM NO_3^- (+N or -N). The net NH_4^+ flux was measured in the uptake solutions (+K+N, N = 200 μM NH_4^+ and K = 200 μM K^+; or -K+N) for 30 min uptake.

Pretreatment	Uptake solution			
	+K+N		-K+N	
	($\mu mol\ g^{-1}FW\ h^{-1}$)			
+K+N	4.97 ± 0.45	d*	5.92 ± 0.63	cd
-K+N	6.36 ± 0.18	cd	8.19 ± 0.64	abc
+K-N	8.06 ± 0.34	abc	9.76 ± 1.40	a
-K-N	8.65 ± 0.91	ab	7.00 ± 0.85	bcd

* For comparing all possible pairs of treatment means (±se), Duncan's Multiple Range Test were performed, separately, on the data of net NH_4^+ flux. Means having a common letter are not significantly different at the 5% significance level for small letters.

when NH_4^+ was present (Table 13). In these -K plants the presence or absence of NH_4^+ during pretreatment caused only a much smaller effect (compare fluxes for -K+N and -K-N pretreatments). Clearly, the presence of NH_4^+ in the uptake solution caused a large reduction of $^{86}Rb^+$ (K^+) uptake, regardless of the pretreatments.

The net NH_4^+ fluxes were reduced by 3 days of pretreatment with NH_4^+ in all treatments (Table 14). Removing K^+ from pretreatment solutions caused small increases in NH_4^+ uptake as they had for $^{86}Rb^+$ (K^+) uptake, but these differences were not significant at the 5% level of probability. The presence of K^+ in the uptake solutions caused statistically non-significant reductions in NH_4^+ uptake in all pretreatments except -K-N when NH_4^+ uptake actually decreased. Here again, however, the difference was not statistically significant.

7.3.2. Experiment II. Effects of NH_4^+ provision during growth, and of K^+ and NH_4^+ in pretreatment and uptake solutions on $^{86}Rb^+$ (K^+) influxes.

The effects of three factors (N provision during the growth period, 3 d of K^+ and NH_4^+ pretreatment, and the presence or absence of NH_4^+ in the uptake solution) on $^{86}Rb^+$ (K^+) uptake were examined in Exp II. $^{86}Rb^+$ influx was increased in virtually all treatments by increased levels of NH_4^+ provision during the growth period (see Figs. 40A and 40B). In those experiments where NH_4^+ was present during influx measurement the noted positive effect of NH_4^+ pretreatment was reduced or absent at the highest level of NH_4^+ (100 μM) but was still pronounced between 10 and 50 μM NH_4^+. As in Experiment I, provision of NH_4^+ during the 3 d pretreatment caused the greatest increase of $^{86}Rb^+$ (K^+) influx in low K^+

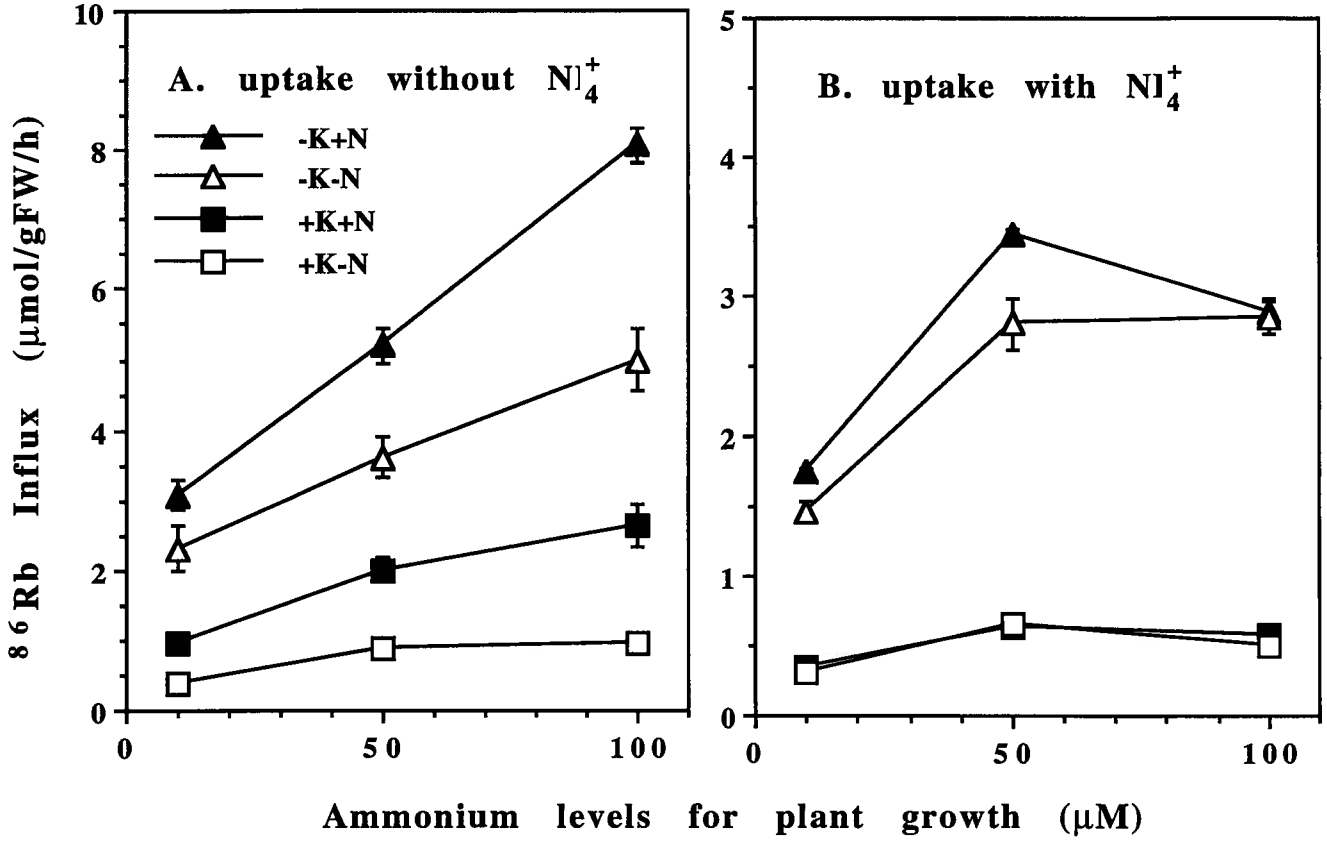


Figure 40. Effects of NH_4^+ in the growth media, pretreatment and uptake solutions on $^{86}Rb^+$ influx. G10, G50, and G100 plants were pretreated for 3 days in four solutions including +K+N (closed squares), +K-N (open squares), -K+N (closed triangles), -K-N (open triangles). The $^{86}Rb^+$ influxes were measured in MJNS containing 200 μM K^+ without NH_4^+ (Fig. 40A) or with 100 μM NH_4^+ (Fig. 40B). Data points are the average of three replicates with ±se as vertical bars.

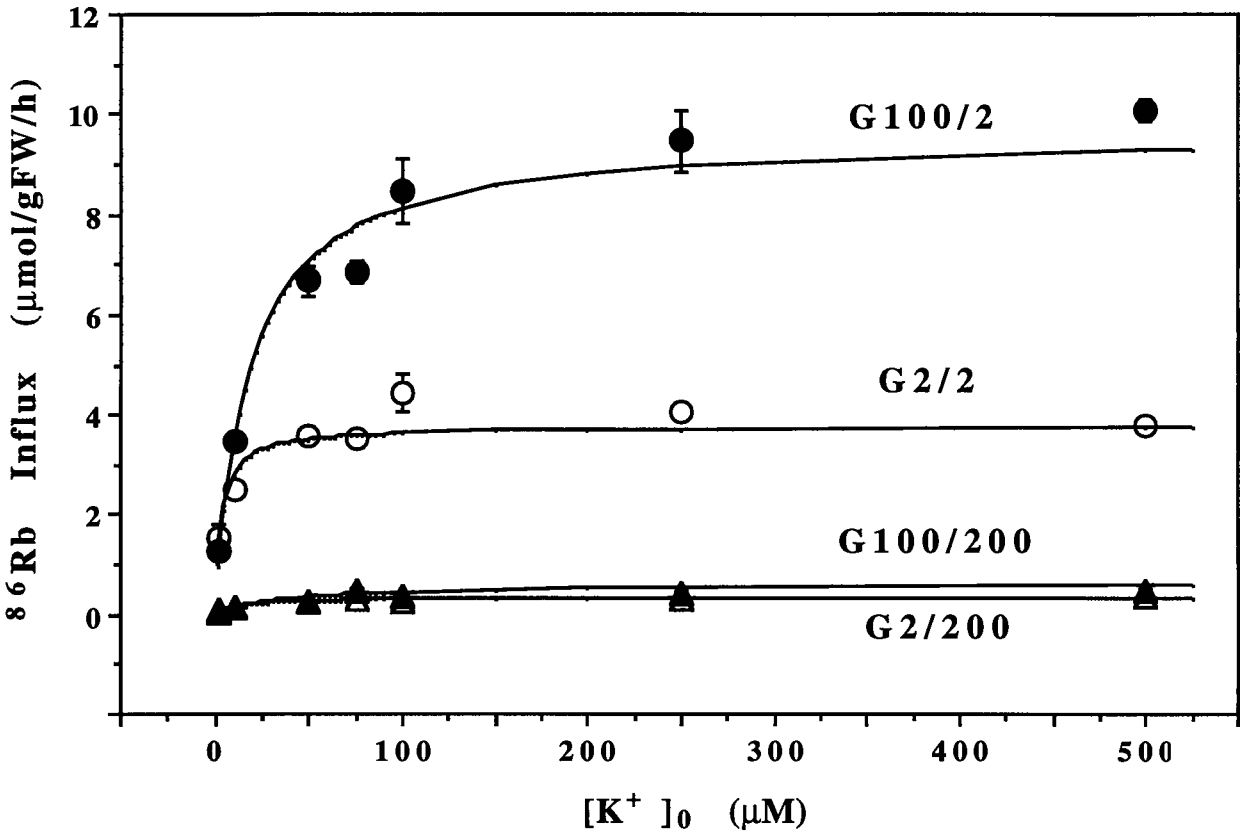


Figure 41. Effects of NH_4^+ and K^+ in growth media and uptake solutions on $^{86}Rb^+$ influx. The $^{86}Rb^+$ influxes of G2/2, G2/200, G100/2, G100/200 plants were measured for 10 minutes in $^{86}Rb^+$ labeled MJNS, containing 2, 10, 50, 75, 100, 250 or 500 μM K^+, respectively, plus 2 μM NH_4^+ for G2/2 (open circle) and G2/200 (open triangle), or plus 100 μM NH_4^+ for G100/2 (closed circle) and G100/200 (closed triangle). Data points are the average of three replicates with ±se as vertical bars.

plants when NH_4^+ was absent from the uptake solutions (Fig. 40A) and the least effect in high K^+ plants in the presence of NH_4^+ during uptake (Fig. 40B). However, as in Experiment I, the presence of NH_4^+ during flux measurements, reduced $^{86}Rb^+$ (K^+) influx in all treatments. Again, removing K^+ from the pretreatment solution caused increased $^{86}Rb^+$ (K^+) influx, and this effect was more pronounced when adequate N was provided (compare squares and triangles to note the K^+ effect, and closed and open triangles to note the N effect).

7.3.3. Experiment III: Effects of NH_4^+ provision during growth, and presence in uptake solution upon influx isotherms for $^{86}Rb^+$ (K^+).

Figure 41 presents the ^{86}Rb influx isotherms for plants grown under G2/2, G2/200, G100/2 and G100/200 conditions. The data were fitted to Michaelis-Menten equations. The kinetics of ^{86}Rb uptake were influenced by the provision of both NH_4^+ and K^+ during three weeks growth. The ^{86}Rb influx curves for G2/200 and G100/200 plants (grown in higher external K^+) revealed a low V_{max}, 0.34 and 0.59 μmol $g^{-1}FW\ h^{-1}$, respectively. By contrast, plants grown in low K (2 μM), exhibited much higher V_{max} value (3.74 and 9.58 μmol $g^{-1}FW\ h^{-1}$ for G2/2 and G100/2 plants, respectively). As in the previous experiments the provision of NH_4^+ during the growth prior to influx measurements caused a significant positive effect; V_{max} for ^{86}Rb (K^+) influx was increased ~3 fold. The estimated values of K_m were also higher for plants grown in higher K conditions (15.02 μM for G2/200 and 38.59 μM for G100/200 plants) than for those grown in low K supply (18.00 μM for G100/2 and 3.47 μM for G2/2). The relationship between estimated kinetic parameters and measured tissue K concentrations clearly indicated the operation of negative feedback inhibition of ^{86}Rb influx.

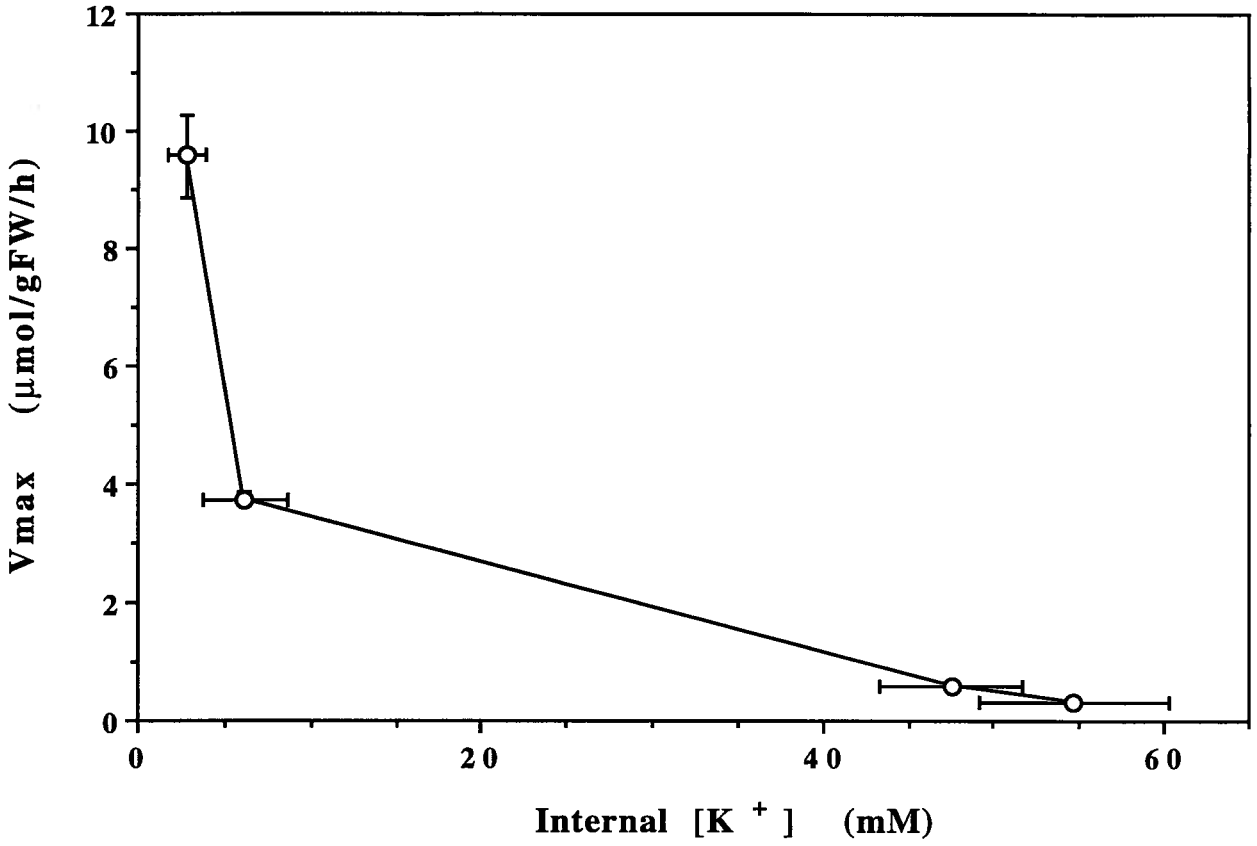


Figure 42. Relationship between estimated kinetic parameter of $^{86}Rb^{+}$ influx (V_{max}) and the assayed roots internal [K^{+}]. The vertical bars are standard errors for V_{max} and the horizontal bars are standard errors for $[K^{+}]_i$.

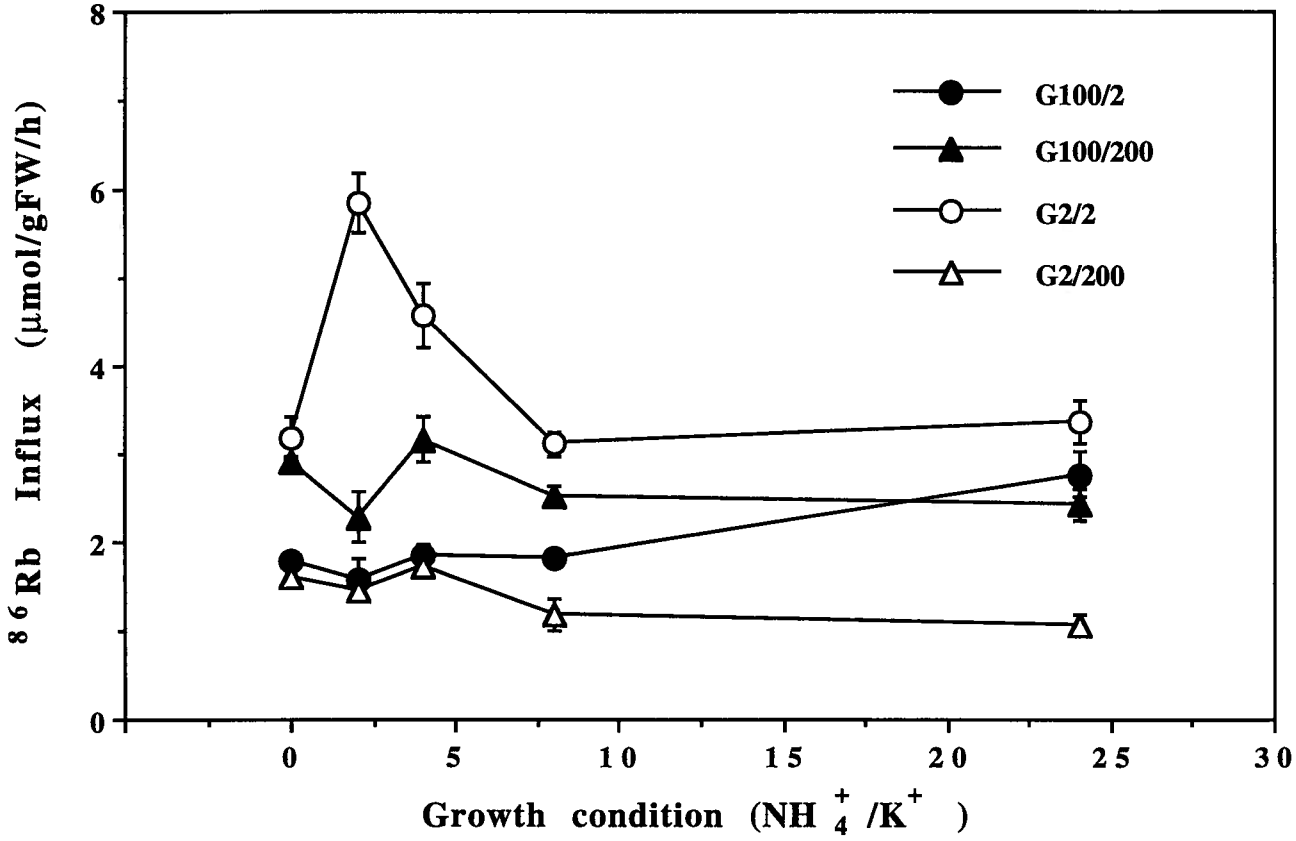


Figure 43. Effect of short-term NH_4^+ pretreatment on $^{86}Rb^+$ influx. Plants were pretreated in 1 mM NH_4^+ plus either 2 μM K^+ for (G2/2 and G100/2) or 200 μM K^+ for (G2/200 and G100/200 plants), respectively, for 0, 2, 4, 8, 24 h. $^{86}Rb^+$ influxes were measured for 10 minutes in $^{86}Rb^+$ labeled MJNS containing 200 μM K^+ and 100 μM NH_4^+. Data points are the average of three replicates with ± se as vertical bars.

Figure 42 showed a strong negative correlation between V_{max} values and internal $[K^+]$ values.

7.3.4. Experiment IV: Effects of NH_4^+ provision during growth, and short-term pretreatment upon $^{86}Rb^+$ (K^+) influx.

When the N status of plants was changed by short-term exposures to NH_4^+, $^{86}Rb^+$ influxes were also altered, as shown previously for 3 days exposures to NH_4^+ (Table 13, Figs. 40 and 41). For plants grown in higher N (G100/2 or G100/200) the $^{86}Rb^+$ influxes were affected little by loading in 1 mM NH_4^+ for various periods (Fig. 43). For plants grown in low N, the results of pretreatment in 1 mM NH_4^+ varied according to the differences in the K status. The $^{86}Rb^+$ influxes of G2/2 plants were greatly increased during the first 4 h pretreatment in 1 mM NH_4^+. In contrast, $^{86}Rb^+$ influxes of G2/200 declined slightly after the first 4 h.

7.3.5. Experiment V: Effect of NH_4^+ concentrations present in uptake solution upon influx isotherms for $^{86}Rb^+$ (K^+).

To further understand the inhibitory effect of NH_4^+ in uptake solutions, $^{86}Rb^+$ influxes were measured at five $[K^+]_o$ levels in the presence of four levels of $[NH_4^+]_o$. Generally, $^{86}Rb^+$ influxes for G100/2 were higher than for G2/2 and G100/200. G2/200 plants had the lowest rates of potassium uptake. Generally, $^{86}Rb^+$ influx decreased with increasing $[NH_4^+]_o$ in the uptake solutions, but the effect on G100/2 is not so evident (Fig. 44). Even at 2 μM $[K^+]_o$, the inhibitory effect of NH_4^+ was evident. Table 15 presents estimated Michaelis-Menten parameters for all $^{86}Rb^+$

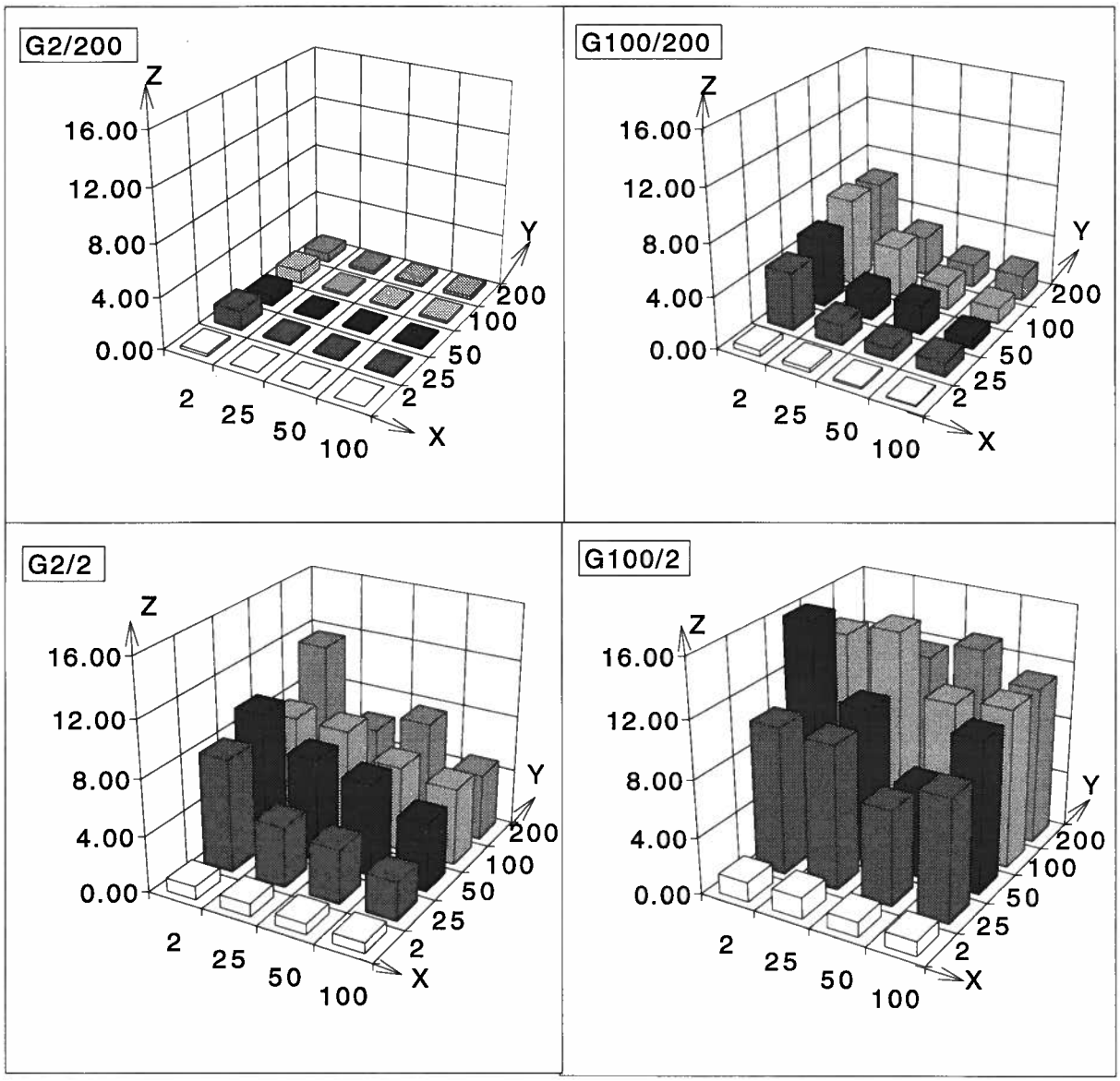


Figure 44. Effects of NH_4^+ and K^+ in growth media and uptake solutions on $^{86}Rb^+$ influx. The $^{86}Rb^+$ influx of G2/2, G2/200, G100/2, or G100/200 plants, respectively, were measured in MJNS containing 2, 10, 50, 100, or 200 μM K^+ plus 2, 25, 50, or 100 μM NH_4^+, respectively. In plots: X = [NH_4^+] (μM), Y= [K^+] (μM), Z = $^{86}Rb^+$ influx (μmol K^+ g^{-1}FW root h^{-1}), respectively.

Table 15. Michaelis-Menten kinetic parameters for $^{86}Rb^+$ influx for four groups of plants (G2/2, G2/200, G100/2 or G100/200). Based on the data of Fig. 44, the parameters were estimated by nonlinear procedure on replicated influx data (n=2).

$[NH_4^+]_o$ (μM)	V_{max} ± se (μmol g^{-1}FW h^{-1})	K_m ± se (μM)	r^2
	G2/2 plants		
2	11.07 ± 1.04	11.82 ± 5.90	0.82
25	7.83 ± 0.78	13.23 ± 7.59	0.79
50	8.27 ± 0.80	23.36 ± 8.73	0.86
100	5.83 ± 0.81	18.39 ± 11.34	0.86
	G100/2 plants		
2	14.97 ± 1.81	8.63 ± 9.05	0.89
25	14.02 ± 1.77	10.99 ± 7..47	0.78
50	15.79 ± 1.80	49.53 ± 16.08	0.93
100	12.34 ± 0.89	11.58 ± 4.66	0.82
	G2/200 plants		
2	1.02 ± 0.13	3.09 ± 1.57	0.17
25	0.41 ± 0.06	105.93 ± 31.03	0.96
50	0.41 ± 0.04	136.18 ± 21.07	0.90
100	0.55 ± 0.12	186.75 ± 70.79	0.98
	G100/200 plants		
2	7.36 ± 0.55	17.60 ± 5.61	0.80
25	4.06 ± 0.54	36.25 ± 15.26	0.81
50	2.18 ± 0.32	17.68 ± 10.73	0.81
100	2.31 ± 0.58	63.88 ± 41.43	0.95

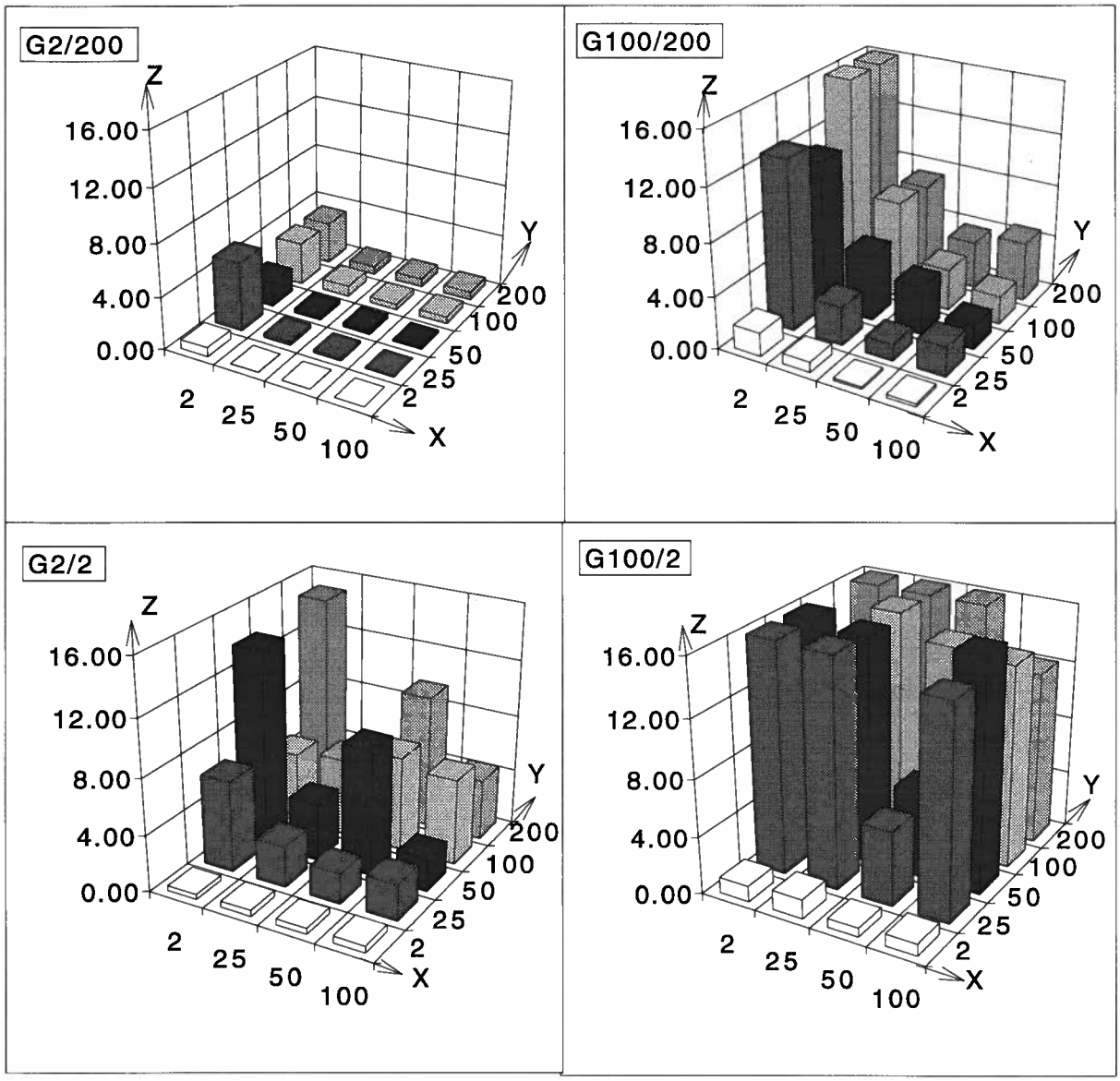


Figure 45. Effects of NH_4^+ and K^+ in growth media and uptake solutions on $^{86}Rb^+$ translocated to shoots. (Details as in Fig. 44) In plots: X = $[NH_4^+]$ (μM), Y= $[K^+]$ (μM), Z = $^{86}Rb^+$ translocated (nmol K^+ g^{-1}FW shoot h^{-1}), respectively.

influxes isotherms. Generally, V_{max} values decreased with increasing $[NH_4^+]_o$ in the uptake solutions. In contrast, K_m values tended to increase with increasing $[NH_4^+]_o$ in the uptake solutions for G2/200 and G100/200 plants (Table 15). However, K_m values remained relatively constant for G2/2 and G100/2 plants. Similar inhibitory effects were true for the translocation of K^+ (^{86}Rb) to shoots (Fig. 45). It was evident that higher rates of ^{86}Rb translocation were associated with growth on sufficient N (G100/200) or insufficient K^+ (G2/2 and G100/2).

In this experiment, plant biomass was recorded in order to make comparisons of the effects of growth conditions. There were statistically significant differences among total fresh and dry weights of plants (G100/200 > G2/200 > G100/2 > G2/2) although the ratios of dry:fresh weight were relatively constant (Table 16). Both fresh or dry shoot weights of plants grown in well-supplied media (G100/200) were significantly higher than for other types of plant. With inadequate supply of either K^+ or NH_4^+, plants (G2/200 or G100/2) plants had smaller biomass but these were still significantly higher than that of G2/2 plants. However, the differences of root weight indicated that K played a more important role in root growth than did NH_4^+ (compare G100/200 to G2/200). When K^+ was adequately supplied, plant roots grew better. Under K^+ stress, NH_4^+ seemed to have little effect on root biomass.

7.3.6. Experiment IV: Effects of K^+ provision during growth and presence in uptake solutions upon influx isotherms for $^{13}NH_4^+$.

The effects of K^+ in the uptake solutions on the $^{13}NH_4^+$ influx were examined using G2/2, G2/200, G100/2, and G100/200 plants (Fig. 46). The

Table 16. Effects of NH_4^+ and K^+ on plant growth. Rice plants were grown in either 2 μM or 100 μM NH_4^+ plus either 2 or 200 μM K^+ (G2/2, G2/200, G100/2 or G100/200, respectively). Each value is the average of 40 sample means (mg per plant) with ± se.

Plants	G2/2	G100/2	G2/200	G100/200
Total FW (mg)	179 ± 5 d	225 ± 6 c	293 ± 14 b	368 ± 3 a
Total DW (mg)	21 ± 1 d	30 ± 1 c	38 ± 2 b	49 ± 2 a
Total D/F	0.13 ± 0.00 b	0.13 ± 0.00Ab	0.14 ± 0.01 b	0.14 ± 0.01 a
St FW (mg)	114 ± 4 c	163 ± 5 b	168 ± 8 b	258 ± 10 a
St DW (mg)	18 ± 1 c	26 ± 7 b	27 ± 1 b	40 ± 2 a
St D/F	0.16 ± 0.00 a	0.16 ± 0.00 a	0.17 ± 0.01 a	0.14 ± 0.01 a
Rt FW (mg)	56 ± 2 c	62 ± 1 c	125 ± 6 a	110 ± 4 b
Rt DW (mg)	4 ± 0 c	4 ± 0 c	11 ± 1 a	9 ± 1 b
Rt D/F	0.06 ± 0.00 b	0.06 ± 0.00 b	0.09 ± 0.01 a	0.09 ± 0.01 a
FW St/Rt	2.03 ± 0.00 c	2.61 ± 0.00 a	1.36 ± 0.00 D	2.38 ± 0.02 b
DW St/Rt	5.43 ± 0.27 b	7.20 ± 0.23 a	2.88 ± 0.24 C	4.92 ± 0.38 b

* For comparing all possible pairs of treatment means, Duncan's Multiple Range Test were performed, separately, on the data of net $^{86}Rb^+$ flux. Means having a common letter are not significantly different at the 5% significance level for small letter.

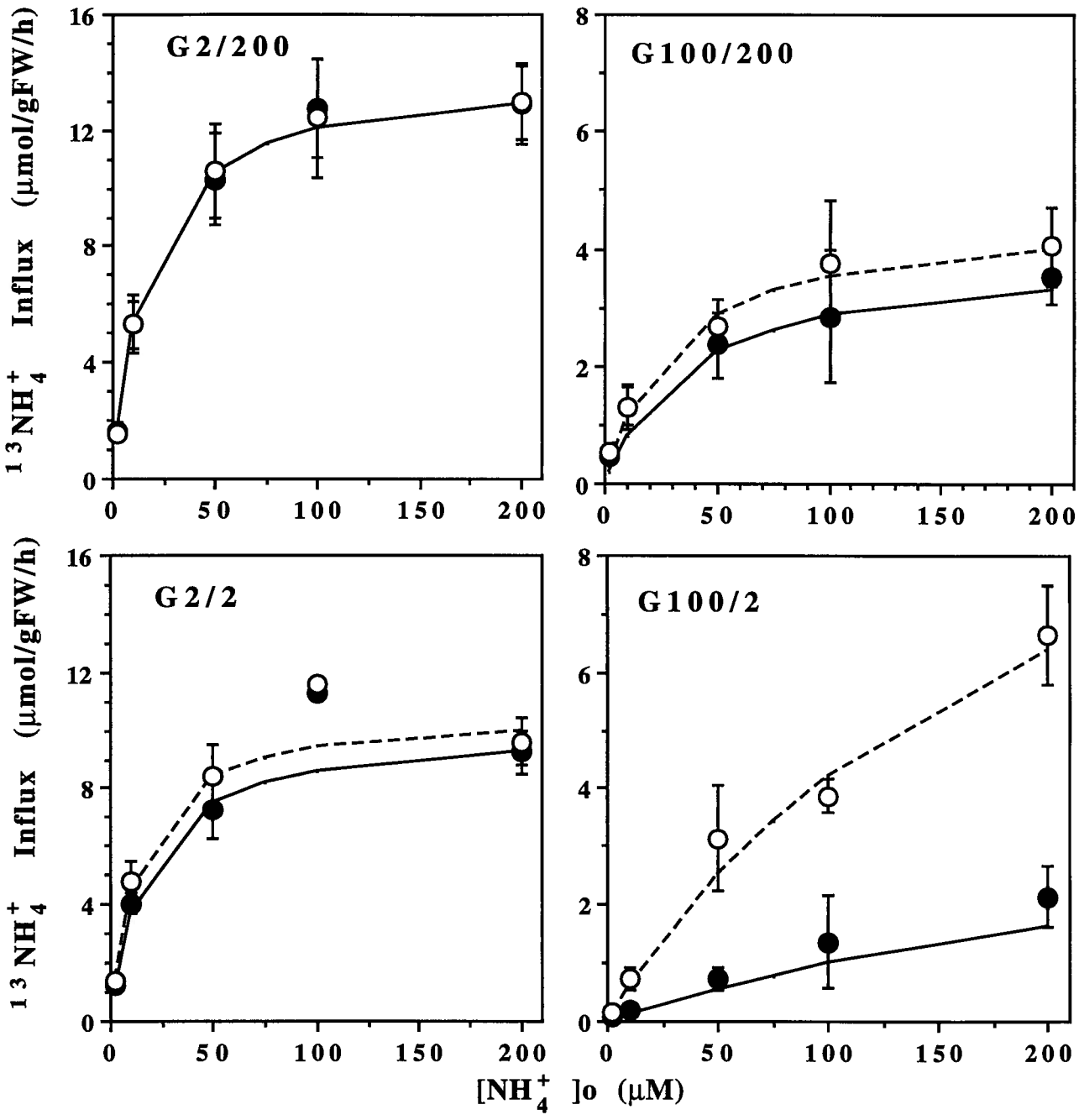


Figure 46. Effects of K^+ in uptake solution on $^{13}NH_4^+$ influx isotherm. Root $^{13}NH_4^+$ influx of G2/2, G2/200, G100/2, or G100/200 plants, respectively, were measured in MJNS containing 100 μM NH_4^+ in the presence of either 2 (open circle) or 200 μM K^+ (closed circle). Predicted isotherms (dashed lines for 0 μM K^+ and solid lines for 200 μM K^+) were calculated from the computed V_{max} and K_m for different plants (Table 17).

Table 17. Michaelis-Menten kinetic parameters for $^{13}NH_4^+$ influx for four plants (G2/2, G2/200, G100/2 or G100/200) derived from influx isotherms based on 2, 25, 50, 100, or 200 μM NH_4^+ with or without 200 μM K^+ (+K or -K). The parameters were estimated by nonlinear procedures on replicated influx data.

Plants	$^{13}NH_4^+$ solution	V_{max} ± se	K_m ± se
G2/2	+K	10.07 ± 0.94	17.40 ± 3.13
G2/2	-K	10.69 ± 0.91	13.62 ± 2.80
G2/200	+K	13.99 ± 2.77	16.36 ± 1.09
G2/200	-K	13.95 ± 2.93	16.22 ± 0.31
G100/2	+K	4.60 ± 1.09	359.36 ± 121.31
G100/2	-K	13.07 ± 2.29	209.11 ± 57.13
G100/200	+K	3.94 ± 0.61	37.89 ± 20.17
G100/200	-K	4.62 ± 0.80	30.56 ± 11.93

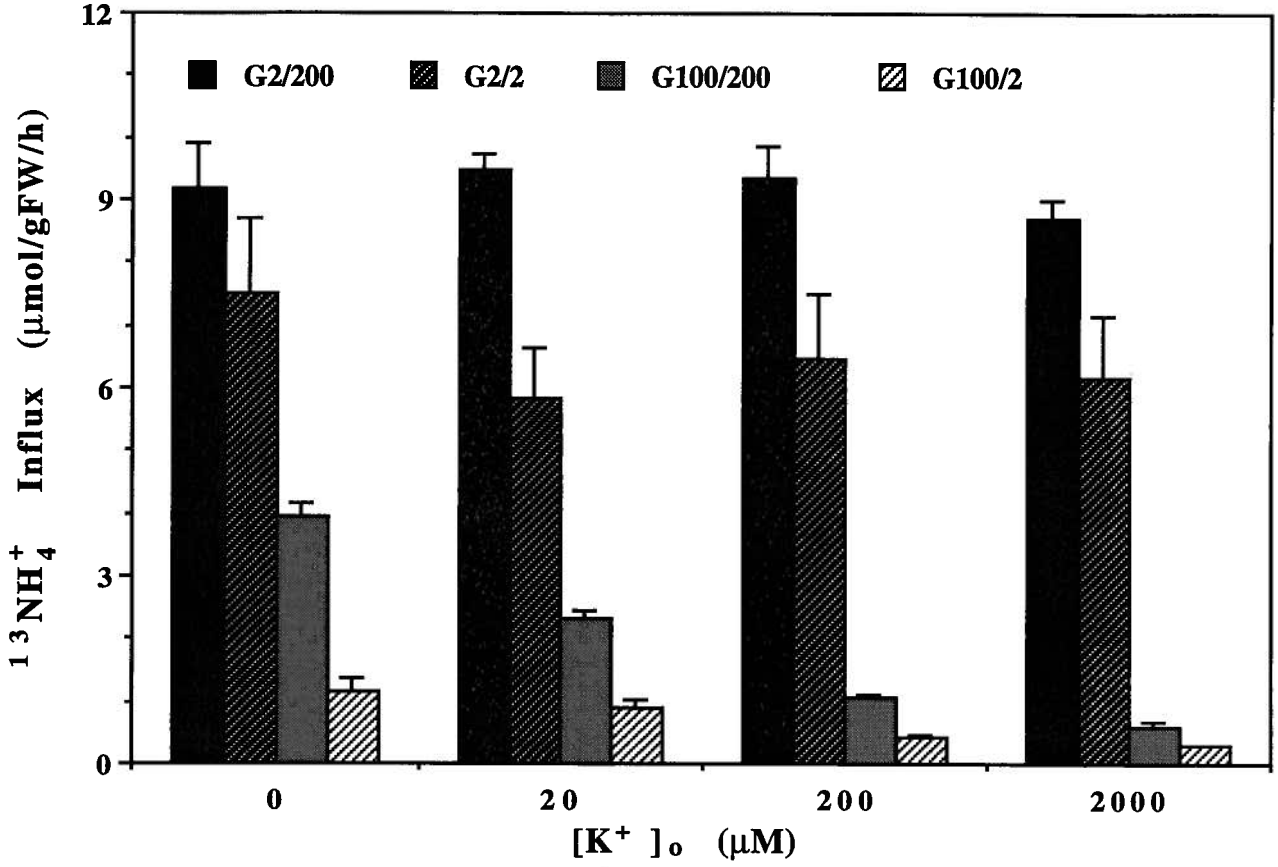


Figure 47. Effects of K^+ in uptake solution on $^{13}NH_4^+$ influx by HATS. Root $^{13}NH_4^+$ influxes of G2/2, G2/200, G100/2, or G100/200 plants, respectively, were measured in MJNS containing 100 μM NH_4^+ in the presence of 0, 20, 200, 2000 μM K^+. Data points are the average of three replicates with ±se as vertical bars.

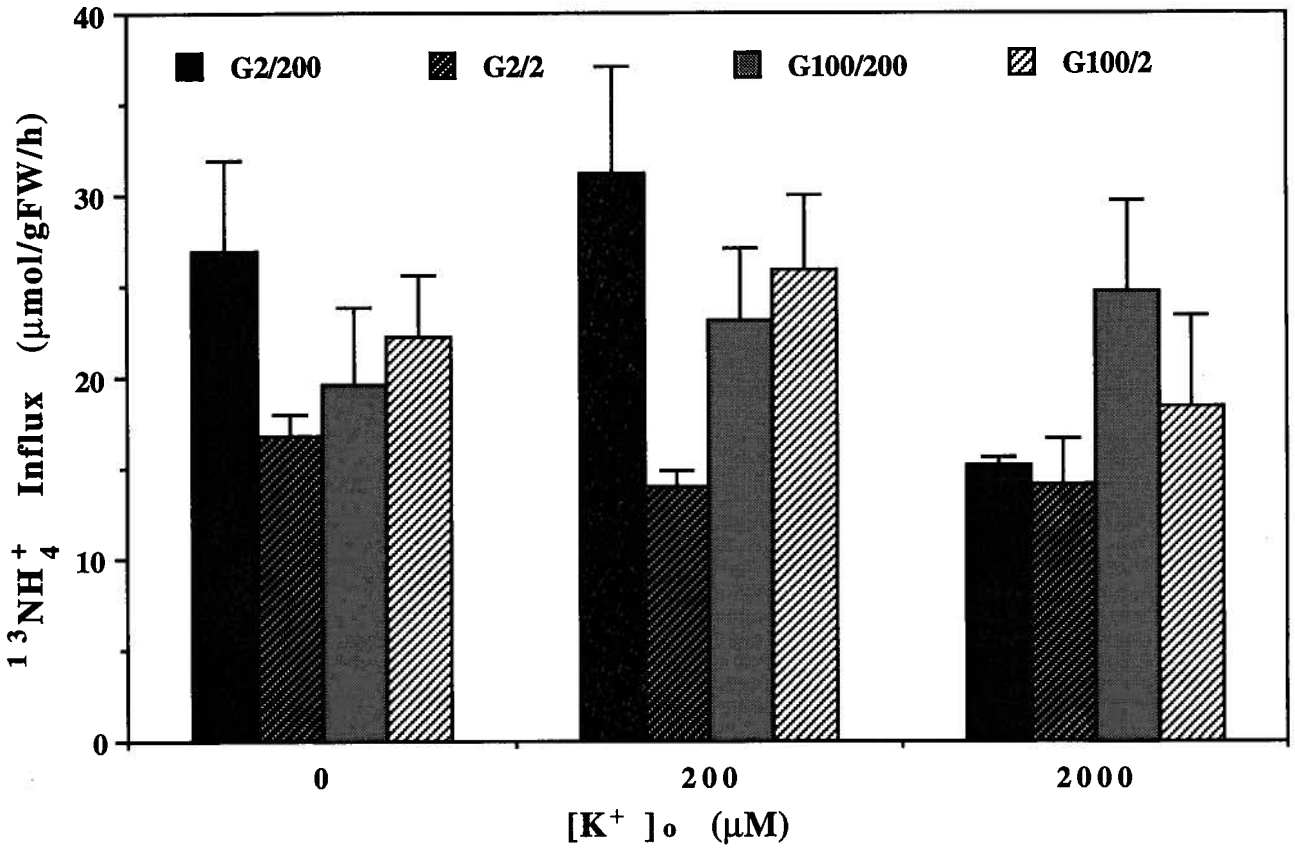


Figure 48. Effects of K^+ in the uptake solution on $^{13}NH_4^+$ influx by HATS+LATS. Root $^{13}NH_4^+$ influxes of G2/2, G2/200, G100/2, or G100/200 plants, respectively, were measured in MJNS containing 1000 μM NH_4^+ in the presence of 0, 200, 2000 μM K^+. Data points are the average of three replicates with ±se as vertical bars.

presence of K^+ in the $^{13}NH_4^+$ uptake solutions failed to significantly reduce $^{13}NH_4^+$ influx except in the case of the G100/2 plants where significant differences were apparent. The estimated influx kinetics also showed the same trends (Table 17). Nevertheless, there were slight reductions of $^{13}NH_4^+$ influx which failed to satisfy statistical evaluation in G2/2 and G100/200 plants. It was noted that plants grown at low N levels had higher $^{13}NH_4^+$ influxes when the K nutrition was adequate during growth (compare G2/200 and G2/2 plants).

When K^+ in the uptake solution was increased from 0, 2, 20, 200, and 2000 μM (Fig. 47), a strong inhibitory effect of K^+ (in the uptake solutions) on $^{13}NH_4^+$ influxes of G100/2 and G100/200 plants was evident. By contrast, $^{13}NH_4^+$ influxes were significantly increased by growth in low N (G2/2 and G2/200) with no effects of K^+ when present in the uptake solutions. The $^{13}NH_4^+$ influxes measured in 10 mM NH_4^+ were not changed significantly although the influxes were lower in the presence of 2000 μM K^+ (Fig. 48).

7.4. Discussion

7.4.1. Plant growth in response to provisions of NH_4^+ and K^+

Both N and K are very important to crop growth and yield. Uptake of K and N, plant dry weight, and paddy yields of rice increased with increasing K and N application rate (Biswas et al., 1987; Ichii and Tsumura H,1989; Fageria et al., 1990). Deficiency of either N or K in the nutrient solution decreased the tissue content of either N or K, influenced

photosynthetic rate and translocation of carbohydrates, caused lower grain weight and therefore reduced rice yield (Grist, 1986; Dey and Rao, 1989). Reduction in photosynthetic rate may be due to impairment of stomatal diffusive conductance and decreased N content/unit leaf area (Dey and Rao, 1989). High tissue K^+ not only promoted CO_2 assimilation, starch formation and the transport of the assimilates but also improved the nitrogen metabolism of the plant and nitrogen use efficiency (Kemmler, 1983; Dibb and Thompson, 1985). K^+ enhanced NH_4^+ assimilation and reduced the toxic effects of NH_4^+ such as stem lesions in tomato or leaf lesion in corn (Ajay et al., 1970; Dibb and Welch, 1976). In a recent paper by Yong et al., (1993) the presence of K^+ in *Arabidopsis*' growth media was responsible for preventing toxic effects of NH_4^+ on root growth. Supplying high levels of K^+ to NH_4^+-N grown plants stimulated shoot growth and more vigorous root growth (Xu et al., 1992).

In the present study the total fresh and dry weights were significantly higher in the sequence of G100/200, G2/200, G100/2 and G2/2 (Table 16). The significant difference between G2/200 and G100/200 indicates the importance of K for plant growth when the N nutrition is adequate. Comparing both fresh and dry weights of roots among four treatments in Table 16, higher K^+ in the growth media produced significantly higher root mass (G100/200 and G2/100) than growth in low K^+ (G2/2 and G100/2), whereas the shoot fresh and dry weights, were not significantly different between G2/200 and G100/2. A greater root mass of seedlings grown in higher K^+ indicated that K^+ may play an important role in facilitating root development (Beaton and Sekhon, 1985; Xu et al., 1992). There was a significant positive correlation between total root weight and K^+ uptake (Table 16). Total root length and dry weight increased as crop growth advanced and N supply increased (Chamuah and Dey, 1988).

However, the root number was negatively correlated with NH_4^+-N uptake in lowland rice (Ichii and Tsumura,1989).

As shown in Table 16, the Shoot:Root ratios for both fresh and dry weights were higher for G100/2 than G200/200 or G2/2. G2/200 plants had the lowest Shoot:Root ratio. It has been reported that N deficiency decreased S/R ratios of seedling plants (Zsoldos et al., 1990). N stress reduces plant growth, particularly shoot growth, through several mechanisms operating on different time scales. The possible signals may be related to N stress-induced changes of abscisic acid and cytokinins (Göring and Mardanov, 1976; Sattelmacher and Marschner, 1978; Chapin et al., 1988a, 1988b; Kuiper et al., 1989). This lower ratio of shoot:root may also due to higher root mass in higher K condition as discussed above.

7.4.2. Effect of plant N status on K^+ ($^{86}Rb^+$) uptake

The nitrogen status of plants had a significant influence on K^+ ($^{86}Rb^+$) uptake. Typically, $^{86}Rb^+$ influxes of G10, G50 and G100 plants were increased with increasing $[NH_4^+]_e$ levels in growth media (Figs. 40A and 40B). The presence of NH_4^+ during the pretreatment period also caused increased $^{86}Rb^+$ influx (Figs. 40A and 40B). $^{86}Rb^+$ uptake by roots exposed to +K+N and -K+N pretreatments were significantly higher than that for +K-N or -K-N pretreatments (Table 13). This positive effect of N status on K^+ uptake may be related to protein synthesis for K^+ transport. The long term regulation of ion uptake probably involves induction or derepression of carrier synthesis. It is known that plants respond to K^+ deprivation rapidly by synthesizing novel polypeptides in the plasma membrane (Fernando et al., 1992) which are believed to form part of the high affinity K^+ transport

system (Glass and Fernando, 1992). When plants were grown in low K (2 μM), with sufficient N supply (100 μM NH_4^+), K^+ ($^{86}Rb^+$) influx was promoted (Figs. 41, 44). However, when the supply of nitrogen was limited during plant growth, the synthesis of K^+ transporters in the cell membrane may be limited. In the present study, when G2/2 plants were pretreated with 1 mM NH_4^+ for 4 hours, more transporters could be synthesized and the $^{86}Rb^+$ influx was significantly increased and remained relative high during the 24 h pretreatment (Fig. 43). This raises an important question concerning the 'induction' of increased NH_4^+ uptake observed when low N plants are first exposure to NH_4^+ (Goyal and Huffaker, 1986; Morgan and Jackson, 1989; Wang et al., 1993b; in Chapter 6). The observation that exposure to NH_4^+ also increased K uptake on a similar time scale indicates that this NH_4^+ effect is not specific as for example the induction of NO_3^- uptake by exposure. Rather, it appears that the so-called 'induction' may be general positive N effect associated with N-depleted plants.

Another possible explanation for the positive effect of NH_4^+ may be due to the effect of N supply on growth rate. The influx of ions into roots may be negatively correlated with the internal concentration of a particular ion, such as Cl^- (Cram, 1973); K^+ (Young et al., 1970; Pitman and Cram, 1973; Glass, 1975; Glass and Dunlop, 1978); NO_3^- (Siddiqi et al., 1992), and SO_4^{2-} (Smith, 1975). Figure 3 showed that the V_{max} for $^{86}Rb^+$ influx was negatively correlated with internal K^+ levels in agreement with previous reports (Glass, 1975; Clarkson, 1983; Pettersson, 1986; Zsoldos et al., 1990). V_{max} decreased and K_m increased exponentially with increased tissue K^+ concentration (Dunlop et al., 1979; Glass, 1976, 1977, 1978). In the present study, the $^{86}Rb^+$ influx was increased in the sequence of G100/2, G2/2, G100/200, and G2/200 (Figs. 41 and 44) and coincides with the sequence of $[K^+]_i$ of these roots. Higher $^{86}Rb^+$ influxes also resulted

from three days pretreatment in minus K^+ solution (Figs. 40A and 40B). Therefore high N supply, resulting in increased plant growth, would cause the opposite effect on tissue [K^+] and K^+ ($^{86}Rb^+$) influx, i.e. a biological dilution effect. This may explain why NH_4^+ supplement to rice plants promotes K stress (Noguchi and Sugawara, 1966), or reduced K^+ concentration of plants (Claassen and Wilcox, 1974; Faizy, 1979; Lamond, 1979).

7.4.3. Effect of NH_4^+ in the uptake solution on K^+ ($^{86}Rb^+$) uptake

Despite the positive effect of NH_4^+ provided during the growth period and the pretreatment period, NH_4^+ has been shown to strongly inhibit the absorption of K^+ in short-term experiments (Bange et al., 1965; Moraghan and Porter, 1975; Breteler, 1977; Munn and Jackson, 1978; Rosen and Carlson, 1984; Scherer et al., 1984). In the present study, $^{86}Rb^+$ influxes were inhibited by the presence of NH_4^+ in the uptake solution (Figs. 40A, 40B and 44, Table 13 and 15). The inhibition of $^{86}Rb^+$ influx increased with increasing [NH_4^+] in the uptake solutions (Table 15). The uptake of K^+ by excised rice roots decreased markedly with increasing concentrations of NH_4^+ in the uptake solution (Scherer et al., 1987). Greater inhibition of K^+ uptake was exerted by 1000 μM NH_4^+ than 100 μM NH_4^+ (Rosen and Carlson, 1984), and the inhibition by 1000 μM NH_4^+ occurred after 90 min treatment and the inhibition by 100 μM NH_4^+ took about 240 min (Jongbloed et al., 1991).

Since this inhibitory effect of NH_4^+ on K^+ ($^{86}Rb^+$) influx is independent of K^+ provision or pretreatments, it is probably exerted on the transport processes at the plasma membrane. It is suggested that certain

solutes are bound to, or associated with, a particular transporter. When an ion of a particular species is attached to this transporter, another similar ion (of the same or a different species) may compete for the same binding site and reducing its uptake. Mixed competitive and non-competitive inhibition between K^+ and NH_4^+ has been reported for tobacco (Scherer et al., 1984) and barley (Dean-Drummond and Glass, 1983). Although NH_4^+ may not always inhibit K^+ uptake competitively, NH_4^+ often has a lower affinity for the carrier than K^+ (Conway and Duggan, 1958; Jongbloed et al., 1991). Likewise, it was found that the K_m for K^+ was increased by NH_4^+ supplementation in ectomycorrhizal fungi (Boxman et al., 1986; Jongbloed et al., 1991).

There was a considerable K^+ efflux induced by NH_4^+ influx during NH_4^+ uptake by roots of corn, wheat or oat (Becking, 1956; Morgan and Jackson, 1989). NH_4^+ markedly inhibits K^+ uptake in many species including wheat (Tromp, 1962), barley (Bange et al., 1965; Meijer, 1970). maize (Rufty et al., 1982) and tobacco (Scherer et al., 1984). It was also reported that exposure of seedlings of Scots pine and Douglas fir to NH_4^+ induced a loss of K^+ (Boxman and Roelofs, 1986; Bledsoe and Rygiewicz, 1986). The need to maintain cation-anion balance may explain some aspects of this inhibitory effect. For example, the presence of monovalent cations (NH_4^+, K^+, Na^+) in the uptake solution depressed $^{45}Ca^{2+}$ influx due to stimulated Ca^{2+} extrusion (Siddiqi and Glass, 1984). Generally plants supplied with NH_4^+-N contain lower concentrations of inorganic cations such as Ca^{2+}, Mg^{2+}, K^+ (Kirkby and Mengel, 1967; Barker and Maynard, 1972; Harada et al., 1968; Moraghan and Porter, 1975; Magalhães and Wilcox,, 1983; Scherer et al., 1984; Siddiqi and Glass, 1984). It was found that K content of white mustard leaves was reduced to near half that of NO_3^--N grown by growth on NH_4^+-N (Kirkby, 1968). Similar competitive

effects were also found in maize and sugar beet when grown on either urea or NH_4^+-N (Beusichem and Neeteson, 1982).

Although NH_4^+ may stimulate the leakage of K^+, it may not be the main mechanism responsible for the inhibition of K^+ influx. It is well known that NH_4^+ uptake is associated with H^+ efflux and acidification of growth media (Pitman, 1970; Riley and Barber, 1971; Pitman et al., 1975; Revan and Smith, 1976; Haynes and Goh, 1978; Bagshaw et al., 1982; Marschner and Römheld, 1983; Nye, 1986; Youssef and Chino,1989; Jongbloed and Borst-Pauwels, 1990; Chaillou et al., 1991). It is a common practise to add base to neutralize the H^+ generated in growth media (Barker et al., 1966; Rufty et al., 1983; Thoresen et al., 1984; Vessey et al., 1990; Wang et al., 1993b). It was estimated that the uptake of 1 mol NH_4^+ required the excretion of 1.33 mol H^+ and 0.33 mol K^+ entered root cells (Raven, 1985). Further, K^+ uptake is intimately associated with active H^+ efflux (Mitchell, 1970; Glass et al., 1981). The K^+:H^+ exchange stoichiometries were almost consistently greater than 2:1 (Glass and Siddiqi, 1982). Last, but not least, the efflux of K^+ was not significant in uptake regulation (Glass, 1983) compared to the importance of K^+ influx (Johansen et al., 1970; Yong and Sims, 1972). Since the presence of NH_4^+ in solution inhibited K^+ uptake to a greater extent in K^+-loaded plants than in K^+-starved plants (Rosen and Carlson, 1984), the efflux of K^+ may not affected by the addition of NH_4^+ (Jongbloed et al., 1991).

7.4.4. Effect of K^+ on NH_4^+ uptake

The uptake of $NH4^+$ by young rice plants, as well as tomato and plum was not competitively affected by the K^+ concentration of the nutrient medium (Mengel et al., 1976, 1978; Rosen and Carlson, 1984) or by plant K

status (Rosen and Carlson, 1984; Scherer and Mackown, 1987). However it was found that the addition of high concentrations of K^+ caused a reduction in methylamine transport rate in *Anacystis nidulans* (Boussiba et al., 1984).

There is a synergistic behavior between N and K in the scope of crop growth and production (Mengel, 1989). Plant NH_4^+-N nutrition was improved by supplying K^+ (Mengel et al., 1976; Dibb and Thompson, 1985). For example, barley response to increasing N concentrations was dependent on levels of K in the whole plant sample (MacLeod, 1969). The much higher N and K uptake with the higher K supply rate suggested that there might be a complementary uptake effect between NH_4^+ and K^+ (Dibb and Thompson, 1985). Lee and Rudge (1986) found that both K^+ and NH_4^+ uptake were stimulated to the same extent in N-starved roots. In greenhouse tests, K application tended to increase grain N content and total N uptake by rice plants (Chakravorti, 1989). Tomato plants grown in sand culture with high NH_4^+ appeared to display symptoms of NH_4^+ toxicity related to increased ethylene synthesis that declined as K supply increased (Corey and Barker, 1989).

In the present study, $^{13}NH_4^+$ influxes of G100/2, G100/200 and G2/2plants were reduced by the presence of K^+ in the uptake solution. Clearly K^+ was most inhibitory to NH_4^+ influx when plants were N-sufficient (Figs. 46 and 48) and K-deficient, especially at high $[K^+]_o$ (Fig. 47). In the former condition, the NH_4^+ influx would be relative low and probably mediated by the high affinity transport system (Wang et al., 1993b). Studies on rice and tomato showed that K^+ had inhibitory effects but did not compete with NH_4^+ for selective binding sites in the absorption process (Ajay et al., 1970; Dibb and Welch, 1976; Mengel et al., 1976).

7.4.5. Shared transport and different feedback signal?

It is known that at low external concentrations, both NH_4^+ and K^+ transport depend on a source of metabolic energy (Kochian and Lucas, 1982; Hong and Stutte, 1987) and conform to Michaelis-Menten kinetics (Epstein, 1972; Debnam and Levin, 1975; Polley and Hopkins, 1979; Fischer and Lüttge, 1980; Kochian and Lucas, 1982; Lüttge and Higinbotham, 1982; Wang et al., 1993b). The rapidity of the inhibitory effects of NH_4^+ and K^+ on each other observed in the present studies indicated that inhibition probably occurred at the level of membrane transport although this inhibition may not be a competitive one. Similar results were reported for maize roots (Shaff et al., 1993). This suggests that NH_4^+ and K^+ may share a common transport pathway, such as an ion channel (Wang et al., 1992b, 1993b; Shaff et al., 1993) and this hypothesis is supported by molecular evidence. In a recently cloned K^+ channel from *Arabidopsis*, the NH_4^+ conductance was determined to be 30% of the K^+ conductance for the KAT1 K^+ channel (Schachtman et al., 1992).

Uptake of both NH_4^+ and K^+ caused depolarization of plasma membrane electrical potentials (Kochian and Lucas, 1989; Ullrich et al., 1984; Wang et al., 1992b). Since the influx of both cations may be driven by the proton motive force (at high external concentration), diminishing membrane potential may lead to reduced ion uptake by influencing the proton motive force. It has been reported that the depolarization of the plasma membrane by NH_4^+ may increase the K_m for K^+ (Kleiner, 1981; Borst-Pauwels et al., 1971; Roomans and Borst-Pauwels et al., 1977; Jongbloed et al., 1991). However the effect on membrane potential can not explain why NH_4^+ inhibited K^+ uptake in all four nutrient treatments

(G2/2, G2/200, G100/2, G100/200) and K^+ only inhibited NH_4^+ influx at high N/low K plant status.

$^{13}NH_4^+$ influx and its kinetic parameters (V_{max} and K_m) of N-deficient plants (G2/2) were not significantly affected by the presence of K^+ in uptake solution except as noted above for the G100/2 plants. Also the inhibition of K^+ ($^{86}Rb^+$) influx by NH_4^+ was lower when plants were K^+-starved. The uptake of K^+ by excised rice roots decreased markedly with increasing concentrations of NH_4^+ in the uptake solution, while the uptake of NH_4^+ was little affected by the concentration of K^+ in the uptake solution (Scherer et al., 1987). K^+ uptake was suppressed during rapid NH_4^+ uptake by N-starved plants (Tromp, 1962), but K-starvation did not produce the same effect as N-starvation on the transport of NH_4^+ (Tromp, 1962; Lee and Rudge, 1986). This biased inhibitory effect between NH_4^+ and K^+ may suggest that NH_4^+ and K^+ share a common transport pathway, but the regulation signal for these two ions may arise from separate sources. The superior competitive behavior of NH_4^+ over K^+ is similar to the inhibitory effect of NH_4^+ on NO_3^- uptake which has also been linked to the depolarizing effects of NH_4^+ on $\Delta\Psi$ (see Lee and Draw, 1989 for discussion). Yet it is clear that, although K^+ causes a depolarization of $\Delta\Psi$ similar to that caused by NH_4^+, it is not inhibitory to NO_3^- uptake, nor is it as effective inhibiting NH_4^+ uptake. Hence it is unlikely that the inhibitory effect of NH_4^+ is due to membrane depolarization/dissociation of pmf. The basis of NH_4^+ inhibitory effect remains to be resolved.

Chapter 8. GENERAL CONCLUSIONS

This study has identified and characterized the ammonium uptake system in rice roots in terms of cellular compartmentation (Chapter 3), kinetics (Chapter 4), energetics, electrophysiology (Chapter 5) and biochemistry (Chapter 6). The interaction between NH_4^+ and K^+ on the plant growth and ion uptake was also examed (Chapter 7).

Ammonium is absorbed by rice roots in the cation form even at elevated $[NH_4^+]_o$. Newly absorbed NH_4^+ is either stored in the root cell vacuoles or rapidly metabolized to amino acids in roots. Amino acids, but not NH_4^+, are consequently translocated to the shoots. Cytoplasmic $[NH_4^+]$ may range from 3 to 38 mM according to the N provision during growth.

The concentration dependence of NH_4^+ uptake demonstrated that, at least, two individual systems, HATS and LATS, operate at the plasma membrane to transport NH_4^+ into root cells. A saturable pattern of $^{13}NH_4^+$ influxes is due to HATS and a linear relationship between $^{13}NH_4^+$ influx and $[NH_4^+]_o$ is mediated by LATS. HATS and LATS are not only kinetically different, but also different in energy dependence and stoichiometry of membrane potential depolarization.

Significant efflux of NH_4^+ was observed even when plants were grown at lower level of $[NH_4^+]_o$, 2 μM. Efflux increased as $[NH_4^+]_o$ increased from 2 to 100 and 1000 μM, correspoding to 10, 20 30% respectively of influx at these $[NH_4^+]_o$.

NH_4^+ uptake is subjected to negative feedback regulation by both NH_4^+ and its metabolites.The effects of pretreatment with exogenous Gln,

Glu and Asn were found to reduce influx to differing extents. A cascade regulation system is proposed to explain the regulation of ammonium uptake in response to changes of internal NH_4^+ and its metabolites. This involves regulation at many levels, from the whole plant down to the molecular level.

The results of NH_4^+ and K^+ interaction studies at the level of plant growth and uptake gave quite different results. Both cations are essential for plant growth, and utilization of each nutrient is optimized when each is in adequate supply. At the uptake level, pretreatment with NH_4^+ caused a strong stimulation of K^+ uptake, but was inhibitory to K^+ uptake when it was present in the uptake solution. By contrast, K^+ was inhibitory to NH_4^+ uptake only when plants were K^+ starved and N (NH_4^+) sufficient. The inhibitory effect of these cations is probably not due to competition for p.m.f., but to direct effect of these ions on the individual transporters.

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Appendix A. Reported studies on using radioactive isotope ^{13}N

Year	Author	N Species	Objective	Material
Berkeley, U.S.A.				
1940	Ruben et al,	$^{13}N_2$	N_2-Fixation	Non-legume
England				
1961	Nicholas et al,	$^{13}N_2$	N_2-Fixation	Bacteria
Manitoba, Canada				
1967	Campbell et al,	$^{13}N_2$	N_2-Fixation	Microorganism
Michigan, U.S.A.				
1974 & 76	Wolk et al,	$^{13}N_2$	N_2-Fixation	Blue-green algae
1975 & 77	Thomas et al,	$^{13}N_2$	N_2-Fixation	Blue-green algae
1977 & 78(a)	Meeks et al,	$^{13}N_2$	N_2-Fixation	Blue-green algae
1978 (b)	Meeks et al,	$^{13}N_2$	N_2-Fixation	Soybean
1978	Skoukit et al,	$^{13}NO_3^-$,$^{13}NH_4^+$	N assimilation	Tobacco cells
1979	Hanson et al,	$^{13}NH_3$	Translocation	Barley
1979 & 81	Tiedje et al,	^{13}N	Denitrification	soils
1981	Schubert et al,	$^{13}NH_4^+$	N_2-Fixation	Non-legume
Davis, U.S.A.				
1976	Gersberg et al,	$^{13}NO_3^-$	Denitrification	Flooded rice soil
1978	Gersberg et al,	^{13}N	N assimilation	Phytoplankton
1982	Thayer&Huffaker	$^{13}NO_3^-$	NO_3^- transport	Klebsiella
1985	Meeks et al,	$^{13}NO_3^-$	Translocation	Cynobacteria
Lower Hutt, New Zealand				
1981	McCallum et al,		Denitrification	Soils
1983 & 85	McNaughton et al,	$^{13}NO_3^-$,$^{13}NH_4$+	N uptake, Flux	Maize
1984 & 86	Presland et al,	$^{13}NO_3^-$	N uptake, Flux	Maize
Quebec, Canada				
1984	Caldwell et al,	$^{13}NO_3^-$,$^{13}NH_4^+$,$^{13}N_2$	N_2-Fixation	Alfalfa
Vancouver, Canada				
1985	Glass et al,	$^{13}NO_3^-$	N uptake, Flux	Barley
1989	Siddiqi et al,	$^{13}NO_3^-$	N uptake, Flux	Barley
Wantage, England				
1986 (a,b)	Lee et al,	$^{13}NO_3^-$	N uptake, Flux	Barley
Stockholm, Sweden				
1987	Oscarson et al.,	$^{13}NO_3^-$	N uptake, Flux	*Pisum*
1988 (a,b)	Ingemarsson et al.,	$^{13}NO_3^-$	N uptake, Flux	*Lemna*
New York, U.S.A.				
1989	Calderon et al,	^{13}N-glutamine	assimilation	*Neurospora crassa*
Houston, U.S.A.				
1990	Hole et al,	$^{13}NO_3^-$	NO_3^- transport	Maize
Jülich, Germen.				
1992	Wieneke	$^{13}NO_3^-$	NO_3^- transport	Squash

Appendix B Reported values of half-life ($t_{1/2}$) and ion content (Q) of various compartments of root cells.

		Superficial	Free space	Cytoplasm	Vacuole
		$t_{1/2}$			
		(sec)	(min)	(min)	(h)
K^+	onion	15 - 43	3.3 - 7.3	82 - 103	80 - 108
	barley			25 - 75	14 - 30
	barley			29 - 30	11 - 23
Na^+	onion	18 - 19	2.8 - 3.7	18 - 23	326 - 362
	barley			20 - 22	77 - 231
	barley			17 - 25	35 - 390
Ca^{++}	onion	12 - 13	1.3 - 1.5	54 - 56	12 - 30
Mg^{++}	onion	18	3.2	74	49 - 71
Cl^-	onion	18 - 52	9.5 - 17.9	90 - 104	68 - 137
NO_3^-	barley				
	barley				
		Q (μmol g^{-1})			
K^+	onion	0.2 - 1.4	0.3 - 1.0	0.8 - 1.3	72.7 - 73.1
	barley			11.0 - 22.0	28.0 - 95.6
Na^+	onion	<0.1 - 0.8	<0.1 - 0.3	<0.4 - 0.1	34.3 - 34.9
	barley			0.6 - 3.6	37.9 - 76.0
	barley			0.5 - 2.8	25.0 - 46.0
Ca^{++}	onion	1.2 - 1.3	0.4 - 0.6	1.6 - 2.5	5.4 - 5.9
Mg^{++}	onion	0.23	0.05	0.32	11.1 - 11.2
Cl^-	onion	<0.1 - 0.4	<0.1 - 0.1	<0.1 - 0.1	68 - 137
NO_3^-	barley				
	barley				